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**Aspects of the stability of salbutamol sulphate in aqueous solution.**

Hakes, Linda Barbara

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ASPECTS OF THE STABILITY OF SALBUTAMOL  
SULPHATE IN AQUEOUS SOLUTION

Submitted by  
Linda Barbara Hakes. B. Pharm., M.P.S.  
for the degree of Doctor of Philosophy  
of the University of Bath

1980

This research was carried out in the School of Pharmacy and  
Pharmacology, University of Bath under the supervision of  
Mr. B.J. Meakin, B.Pharm., F.P.S.

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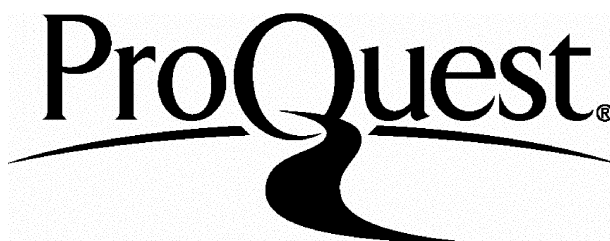
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## SUMMARY

This thesis is concerned with a study of the stability of salbutamol sulphate in aqueous solution together with the effect on its stability, of syrup formulation excipients.

The work necessitated the development of a stability indicating assay. This comprised a preliminary separation on ion-exchange material followed by reversed-phase, ion-pair HPLC assay. A comparison of this technique with the BPC indoaniline colorimetric assay revealed that the latter method seriously overestimates the salbutamol content of degraded solutions.

The degradation of salbutamol sulphate in aqueous solution was studied using pH-statting for pH control in order to avoid the use of buffer salts. The breakdown of the drug was shown to be oxygen sensitive and to increase with drug concentration. However ionic strength did not affect the rate of breakdown.

The pH stability profile of the drug showed a region of minimum stability around pH 9 and maximum stability between pH 3 and pH 4. The slow rate of breakdown in this region precluded a detailed study, but it was shown that the degradation is not a simple acid or base catalysed reaction. Buffer salts were unpredictable in their effects, acetate stabilising the drug whilst phosphate accelerated the breakdown.

The effect of sugars was studied at both pH 7.0 and pH 3.5. Glucose accelerated the degradation of salbutamol sulphate at

pH 7.0 but sucrose had no effect, whilst at pH 3.5, glucose, fructose and sucrose increased the degradation rate to similar extents. This was ascribed to hydrolysis of sucrose at the more acidic pH, liberating the monosaccharides with a potentially "free" carbonyl group. Such a functional group is not available in sucrose because it is utilised in the glycosidic bond.

Further studies to elucidate the nature of the interaction involved the use of glyceraldehyde, glyceric acid, 5-hydroxymethylfurfural and polyhydric alcohols. Attempts were also made to isolate and identify the major breakdown products but since these were unstable definitive identification was not possible. However Figures 55 and 57 show possible structures of isolated compounds.

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## Section 1.

INTRODUCTION1.1 Background

Salbutamol is a sympathomimetic drug which acts almost exclusively on  $\beta_2$  adrenergic receptors. It has a stimulant action, producing bronchodilatation without markedly affecting the cardiovascular system which is controlled principally by  $\beta_1$  receptors (2-5). The discovery that these two pharmacological actions could be separated (6) allowed specific drugs to be developed for the treatment of cardiovascular conditions such as hypertension and bronchial disorders like asthma. Practolol was one of the first cardio-selective drugs (7) and salbutamol an early bronchio-selective (8,9).

Before the introduction of salbutamol in 1969, isoprenaline was the major drug used in the treatment of asthma. However its usefulness was limited by the pronounced tachycardia which it causes in many patients. Salbutamol, therefore, was rapidly accepted as the drug of choice for most asthmatics and consequently has been formulated into virtually all types of pharmaceutical product. Inhalation aerosols provide rapid relief during an asthmatic attack, whilst maintenance therapy can be sustained using tablets or syrup. A crisis of status asthmaticus can be treated by parenteral administration of the drug or by inhalation of the nebulised respirator solution. The selectivity of salbutamol for  $\beta_2$  receptors has led to its use in the management of premature labour when it is generally administered as an intravenous infusion.

Salbutamol therefore provides a good example of the complexities of pharmaceutical formulation and stability testing. The wide range of products must all have a realistic shelf life under all likely storage conditions including those encountered overseas. Foreign markets often require modified formulations for reasons of stability, legislation or simply local preference and so the stability testing of their products is a major concern of all pharmaceutical companies.

### 1.2 Stability Testing

The intrinsic stability of any drug is assessed at an early stage of its development within the company but this does not necessarily bear any relationship to the stability of the final, formulated product. Skilful formulation can significantly increase the shelf-life of an unstable drug, but the reverse situation can also occur, resulting in the rapid degradation of an otherwise stable drug. A common cause of this is the inadvertant incorporation of trace metal ions which then catalyse oxidative processes. These ions may be present as contaminants in an excipient or may originate from equipment used in the handling of the product. This problem has been encountered with several drugs including prednisolone (10), adrenaline (11, 34), labetalol (12) and salbutamol itself (12). Loss of stability may also result from a more specific interaction between drug and excipient and this too has been reported on several occasions (13-17).

The stability of a pharmaceutical product may be assessed under three main aspects, namely, microbiological, physical and



chemical. Microbiological considerations require the prevention of microbial growth and may be tackled in a variety of ways, depending on the nature of the formulation. Some dosage forms, including eye drops and all parenteral formulations, must be supplied in a sterile condition, but unless they are packaged in single-dose units, a suitable preservative must also be included to prevent the growth of micro-organisms which may be introduced during the use of the product. Similar precautions must be taken with products such as oral liquid preparations and topical creams which will be used repeatedly and could provide a suitable environment for microbial growth.

Physical instability may manifest itself in a variety of ways which include precipitation, phase separation, caking, changes in crystal form and changes in dissolution rate. In the case of solution formulations, the most common physical changes are precipitation and colour changes. Both of these should be easy to detect although their prevention may not be so straightforward. In practice, the chemical stability of these formulations tends to present the biggest problem and so receives the most attention. Chemical stability is primarily concerned with the potency of the drug itself although the other ingredients must also be stable under reasonable conditions of storage and use. A well planned stability test will not only assay the active ingredients but also monitor the appearance of breakdown products which may require identification and toxicological study in order to verify that they do not pose a threat to the patient.

All three aspects of stability are commonly assessed by means of accelerated storage tests. These subject the solid drug, its solution or formulated product to more extreme stresses than would be encountered in normal storage. For example, microbial challenge testing, high temperatures, high humidity and high intensity lighting may be used over a short time to mimic the effects of less severe stresses over a long time. Such tests can provide much useful information but their results need to be interpreted with care. Under extreme conditions reactions may be initiated which would not normally be encountered. For instance, many chemical reactions proceed via an "activated complex", which has a much higher energy level than either the reactants or the products. Considerable energy, often in the form of heat, must be supplied before this intermediate can be formed. High temperature storage tests could provide this energy in some cases, where normal storage would not, and so lead to erroneous conclusions about the stability of the compound in question. In the case of formulated products, extra care is needed since the stability of the excipients may be affected to a different extent from that of the drug. If an excipient degrades much more rapidly relative to the drug than it would under normal conditions, a totally different system may result, producing a different spectrum of breakdown products.

For these reasons, preformulation stability studies on the raw drug or simple solutions are usually conducted at higher temperatures and under more extreme stresses than studies on the formulated product. The information gained in the early work, such as the order of reaction, reaction pathways and the

effect of temperature, can then guide the interpretation of the full stability trials.

Pharmaceutical stability is affected by the same factors and conditions which influence chemical reactions in general, the most important ones being temperature, pH, light and humidity. The relative importance of each of these will depend on the nature of the product and of the drug.

Temperature is always a major factor and elevated temperatures are widely used for accelerated storage tests. By establishing the relationship between the rate of breakdown and temperature, it is possible to make predictions of a product's stability at room temperature after conducting short-term tests at higher temperatures. This is commonly achieved by means of isothermal experiments and the Arrhenius equation (Section 1.3), although non-isothermal methods have been advocated (18-20) and at least one group of workers has developed a nonisothermal-isothermal technique (21).

Light can initiate degradative processes by exciting drug molecules to a higher energy state. The additional energy so acquired may enable the molecules to overcome the energy barrier of the reaction and so chemically degrade. In many cases the process, once initiated, can continue in the dark. This is known as autoxidation and is a characteristic of many natural products such as oils, fats and waxes. The reaction is able to continue in the absence of light because reactive species such as free radicals are produced and these perpetuate the breakdown. Such reactions are easily prevented by packing the product in an amber-glass bottle or keeping it in the dark.

The effect of pH is much more complex and a simple relationship between pH and stability does not always exist. Chemical reactions may be acid, water or base catalysed and the state of ionisation of the various chemical entities also plays an important role. Phenols, for instance, are much less stable in alkaline solutions, when they are present as the highly reactive phenoxide ion, than in acidic conditions where they are unionised (22).

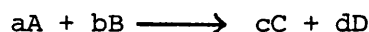
Humidity is important when considering dry products such as powders, tablets, capsules or syrup granules which are reconstituted before use. Powder flow properties, tablet hardness and friability, and dissolution rates in general are easily affected by the level of moisture in the atmosphere. Moisture can also lead to hydrolysis, a common mechanism of drug breakdown and in sensitive products a small increase in moisture content can cause considerable loss of drug potency. Hydrolysis of less sensitive drugs can often be kept to a minimum by careful selection of a suitable pH although this may be restricted by the physiologically acceptable pH range for the type of product. Eye drops and intramuscular injections, for example, can cause severe discomfort if they are far removed from physiological pH and should, ideally, be buffered to pH 7.4. Pharmaceutical formulation is therefore influenced by many, often conflicting considerations which must be carefully balanced before an acceptable, stable product can result.

### 1.3 Reaction Kinetics

Stability studies involve the measurement of rates of

reaction and their comparison under varying conditions.

For the general reaction;



the rate of reaction may be determined by measuring the loss of reactants from the system or the appearance of products. The actual numerical value of the rate will depend on the component measured and may be expressed as  $\frac{-d[A]}{dt}$ ,  $\frac{-d[B]}{dt}$ ,  $\frac{d[C]}{dt}$  or  $\frac{d[D]}{dt}$  where  $[A]$  represents the concentration of species A, etc. and  $t$  is time. It is inconvenient for the rate to be dependent on its method of determination, but by introducing the stoichiometry of the reaction into the expressions, the rate can be rendered independent of the component studied.

$$\text{i.e.} \quad \text{rate} = -\frac{1}{a} \frac{d[A]}{dt} = -\frac{1}{b} \frac{d[B]}{dt} = \frac{1}{c} \frac{d[C]}{dt} = \frac{1}{d} \frac{d[D]}{dt}$$

An alternative view of reaction rate is to regard it as a function of the concentration of all the reactants. Thus, for the general reaction above,

$$\text{Rate} = k[A]^\alpha [B]^\beta \quad \text{where } k = \text{rate constant}$$

$\alpha$  is called the order of reaction with respect to A and  $\beta$  the order with respect to B. The overall order of reaction,  $n = \alpha + \beta$ . In many cases  $\alpha = a$  and  $\beta = b$  and the order is said to be the same as the molecularity of the reaction. However, this is not always the case, particularly in complex reactions which proceed in stages or where the solvent participates in the reaction but does not effectively change in concentration. The order of reaction must therefore be determined experimentally and not assumed from the stoichiometry of the reaction. In simple reactions  $n$  has an integral value

seldom greater than 2. Zero order reactions, where the rate is a constant, independent of reactant concentration, are sometimes encountered in gas phase reactions, particularly those which are surface-catalysed. Reactions in solution are seldom zero order, with the exception of a few enzyme-catalysed reactions.

### First Order Reactions

In this case the reaction rate is proportional to the concentration of a single reactant, i.e.  $-\frac{dC}{dt} = kC$  where  $C$  is the reactant concentration at time  $t$  and  $k$  is the rate constant.

This can be rearranged to

$$-\frac{dC}{C} = kdt \quad \dots (1)$$

which, on integration, yields

$$\ln C_0 - \ln C = kt \quad \text{where } C_0 = \text{concentration at zero time}$$

A graph of  $\ln C$  against  $t$  is therefore linear with a slope of  $-k$ .

### Second Order Reactions

A reaction may be second order in one of two ways. It may be second order with respect to one reactant, i.e.

$$-\frac{dC}{dt} = kC^2 \quad \dots (2)$$

or it may be first order with respect to two different reactants, i.e.

$$-\frac{dC}{dt} = kC_A C_B$$

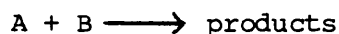
In the first case,

$$-\frac{dC}{C^2} = kdt$$

Integrating, 
$$\frac{1}{C} - \frac{1}{C_0} = kt \quad \dots (3)$$

A plot of  $\frac{1}{C}$  against  $t$  is therefore linear with a gradient equal to  $k$ .

The second case is more complicated



Let the initial concentrations of A and B be  $a$  and  $b$  respectively, and let  $x$  be the amount of each to react in time  $t$ .

$$\frac{dx}{dt} = k(a-x)(b-x) \quad \dots (4)$$

Integration of this equation produces

$$k = \frac{1}{t(a-b)} \cdot \frac{\ln b(a-x)}{a(b-x)}$$

The situation is simplified if  $a$  and  $b$  are equal, that is the initial concentrations of A and B are identical. Equation (4) then becomes

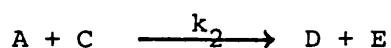
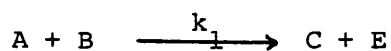
$$\frac{dx}{dt} = k(a-x)^2$$

which integrates to

$$k = \frac{1}{t} \frac{x}{a(a-x)} \quad \dots (5)$$

A further simplification is achieved if one reactant is present in sufficient excess that its concentration does not effectively change over the course of the reaction. The kinetics then approximate to first order with respect to a single reactant. The resultant rate constants are termed pseudo-first order. Higher orders of reaction are possible but are seldom encountered. It is much more usual for the overall reaction order to be non-integral due to the complex nature of the reactions occurring in the system. This is particularly common in oxidation processes where free radicals are often involved

(Section 1.4), although oxidations are sometimes claimed to be zero-order (23). It has been assumed, so far, that only one reaction takes place between the reactants but in practice several reactions may be possible and the products may then react further. This leads to a situation where consecutive, parallel or opposing reactions need to be considered. In order to apply detailed kinetics to such systems, the various components need to be measured independently. This requires knowledge of the nature of both reactants and products as demonstrated by the following pair of reactions.



$$\frac{d[A]}{dt} = -k_1 [A] [B] - k_2 [A] [C]$$

$$\frac{d[B]}{dt} = -k_1 [A] [B]$$

$$\frac{d[C]}{dt} = k_1 [A] [B] - k_2 [A] [C]$$

$$\frac{d[D]}{dt} = k_2 [A] [C]$$

Detailed theoretical treatments of both simple and complex reaction kinetics can be found in the standard texts on chemical kinetics (24-26). However, in many pharmaceutical applications the nature of the products is not known and so only elementary kinetics can be applied.

#### Determination of Reaction Order

The simplest possible expression is always desirable and so first or second order kinetics should be applied initially to test whether they adequately describe the system. The easiest



way of doing this is to plot a graph of  $\ln C$  against  $t$  (first order) or  $1/C$  against  $t$  (2nd order). If a linear relationship down to at least 25% residual concentration is found in either of these cases, the relevant kinetics can be applied although this result should be confirmed by performing experiments with different starting concentrations. For instance, a true first order reaction will have the same rate constant regardless of the initial reactant concentration. However, if, as often happens, the graph is not linear, the reaction orders must be determined by alternative means.

One method is to determine the half-life of the reaction at different starting concentrations. The half-life is the time taken for the concentration to fall to half its initial value. It can be shown (25) that

$$t_{1/2} = \frac{2^{(n-1)} - 1}{(n-1)ka^{(n-1)}} \quad \dots (6)$$

and therefore  $t_{1/2} \propto \frac{1}{a^{(n-1)}}$

$$\therefore t_{1/2} = \frac{P}{a^{(n-1)}} \quad \text{where } P = \text{proportionality constant}$$

$$\therefore \log t_{1/2} = \log P - (n-1) \log a \quad \dots (7)$$

A graph of  $\log t_{1/2}$  against  $\log a$  should therefore be a straight line of gradient  $(1-n)$ . If the graph is non-linear, a change of order with concentration is indicated, caused by a change in reaction mechanism.

An alternative approach is that of van't Hoff's differential method

$$\begin{aligned} \text{Rate} &= kC^n \\ \log(\text{rate}) &= \log k + n \log C \end{aligned}$$

The rate at different concentrations may be measured by taking tangents to the concentration-time curve of the reaction. The logarithms of these values may then be plotted against  $\log C$  to give a graph of slope  $n$ . Again, the method assumes that the order remains constant irrespective of reactant concentration.

Other, more complex methods have been derived but they tend to require a full knowledge of the reaction products, a situation which is seldom possible in pharmaceutical applications. Even when the reaction under investigation is found to have a non-integral order, the reaction often appears to follow simple kinetics over at least the first one or two half-lives and rate constants can be calculated on this basis. For example, many reactions produce a reasonably linear semi-logarithmic plot even though they are not truly first order. The rate constants calculated from this treatment are therefore only apparent first order constants but provide a useful means of comparing reaction rates.

#### Effect of Temperature

An increase in temperature frequently causes a very marked increase in reaction rate. This effect is described by the Arrhenius relationship (Equation 8)

$$k = Ae^{-E_a/RT} \quad \dots (8)$$

where  $A$  = constant (frequency factor)

$E_a$  = activation energy

$R$  = gas constant

$T$  = absolute temperature

The frequency factor,  $A$ , is a constant related to the number of molecular collisions occurring and leading to reaction.

A similar expression (Equation 9) has been derived from a thermodynamic approach, using the activated-complex or transition state theory of reaction rates

$$k = \frac{RT}{Nh} e^{\Delta S^*/R} e^{-\Delta H^*/RT} \quad \dots (9)$$

$N$  = Avogadro's number

$h$  = Planck's constant

$\Delta S^*$  = standard entropy of activation

$\Delta H^*$  = standard heat of activation

Bunnett (27) has developed the theory to produce expressions for  $\Delta H^*$ ,  $\Delta S^*$  and  $\Delta G^*$ , the heat, entropy and free energy of activation respectively. He has demonstrated that in practice  $\Delta H^*$  and  $E_a$  are very similar in value but some workers prefer to use the thermodynamic parameters, which are more relevant to complex reactions (28).

The principal difference between the collision and transition-state theories of kinetics relates to the complexity of the reactants. In the simpler, collision theory, molecules are believed to react if they collide with sufficient energy. Not all collisions will reach this threshold energy level and so only a fraction of collisions actually results in chemical reaction.

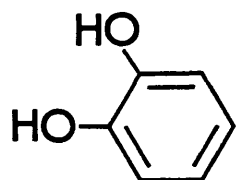
This theory works well for simple molecules such as hydrogen iodide but when the reactants are more complex the theory predicts

higher reaction rates than are observed. This is due to the fact that orientation plays a major role in these reactions and the energy of the molecules must be correctly distributed over the various bonds before reaction will take place. The transition-state theory developed from this and it proposes that a chemical reaction between two or more molecules proceeds via an activated complex which then breaks down to form the reaction products. This complex has a higher free energy than normal molecules and has only a transitory existence. The activation energy of the reaction is then defined as the difference in energy between the activated complex and the reactant molecules (24-27).

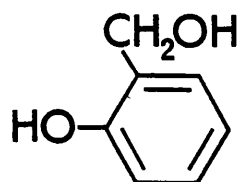
The application of reaction kinetics can therefore assist the formulatory pharmacist to devise a stable product. However, this approach can only be used to the best advantage when the mechanism of drug breakdown is known and this is seldom the case during the early development of a new drug. In this situation, useful information can often be gained from well known, chemically related compounds.

#### 1.4 Stability of Adrenaline and Other Sympathomimetic Drugs

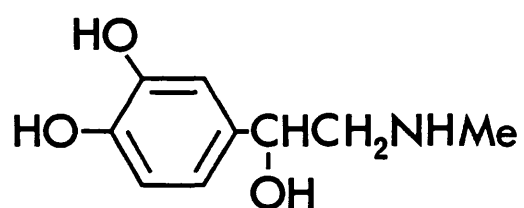
Salbutamol is structurally related to adrenaline and the catecholamines although the catechol group has been modified in salbutamol, making it a saligenin derivative (Fig. 1). Apart from altering the pharmacological activity of the molecule, this modification also confers greater chemical stability on the compound, compared to the catecholamines. The catechol group is very sensitive to oxidation and this sensitivity



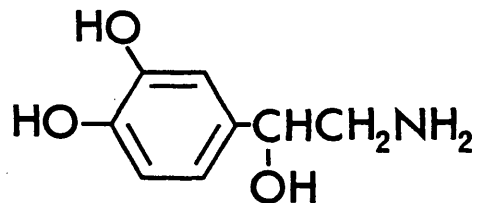
Catechol



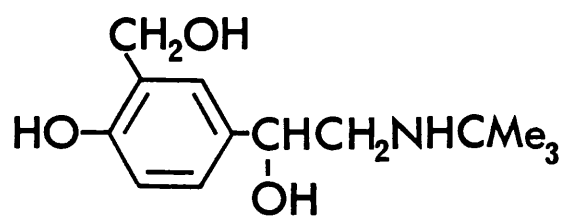
Saligenin



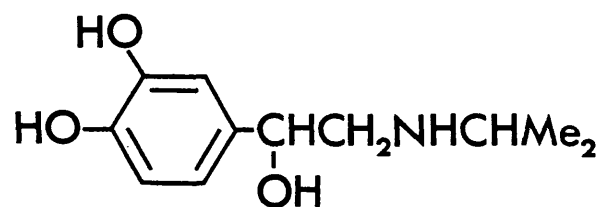
Adrenaline



Noradrenaline



Salbutamol

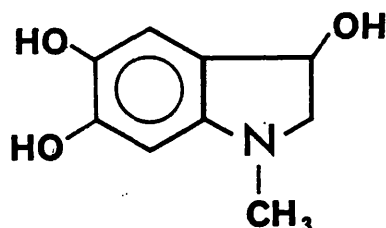


Isoprenaline

Fig. 1. Catechol, saligenin and some biologically active derivatives.

characterises both the natural compounds, adrenaline and nor-adrenaline, and the synthetic drugs such as isoprenaline. Aqueous solutions of adrenaline quickly acquire a pink colour on standing and this has been the subject of considerable research (29-35) as well as posing a formidable problem for pharmacists wishing to prepare acceptable adrenaline formulations for medicinal use.

Oxygen plays a major role in the degradation of adrenaline and similar catecholamines, and oxygen uptake has often been used as a means of following the course of the reaction (11, 36). The nature of the oxidation products formed from adrenaline varies with the pH of the solution. In alkaline solution, oxygen uptake is accompanied by the production of a transient yellow-green fluorescence due to adrenolutin



which rapidly disappears leaving a yellow, then turbid brown solution (29). At pH 6-8 the solution turns red on exposure to air after a preliminary induction period and then darkens due to the formation of polymeric melanins (29). Chaix *et al.* (11) have proposed that this reaction is catalysed by heavy metal ions. In the absence of added metal ions they found that oxygen uptake by adrenaline solutions was very low and suggested that this uptake was due to trace metal contamination. However, at pH 5, true autoxidation in the absence of metal ions has been

demonstrated by Sokoloski and Higuchi (37) who added potassium cyanide to the reaction mixture, to complex heavy metals.

Most metal ions, particularly copper, have been shown to catalyse the oxidation of adrenaline (11,34-37) but iron appears to have a unique role. Inorganic ferric ions alone form a stable violet complex with adrenaline which does not oxidise further at pH 7 (11) but in the presence of EDTA efficient catalysis of adrenaline breakdown occurs (38). Formation of a stable ferric-EDTA complex was proposed, comparable to the natural iron-protein complexes like ferritin or ferricytochrome found in biological systems.

The probable mechanism of adrenaline oxidation under these conditions is given in Fig. 2 (29-31). The red colouration which develops is due to adrenochrome, which itself catalyses the oxidation further by acting as a hydrogen carrier (33).

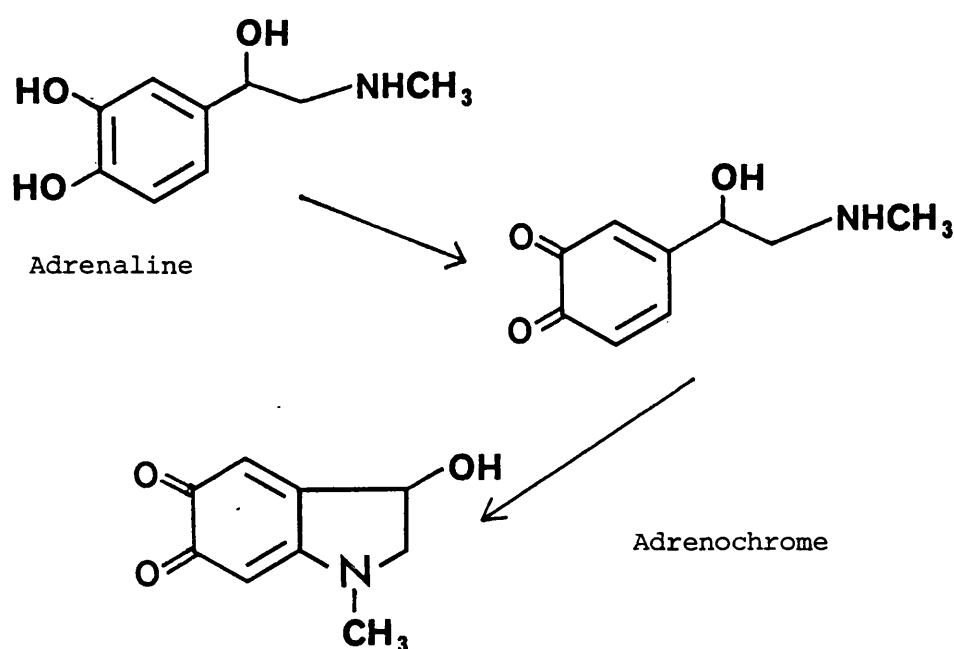


Fig. 2.

The reaction sequence is believed to be mediated by free radicals since a lag time is demonstrated followed by a first order relationship (28). At lower pH, Sokoloski and Higuchi did not detect a lag phase but suggested that this was due to the increased reactivity of the intermediate radical. They concluded that none of the gross oxidation products had a significant catalytic effect (37).

Studies on related compounds such as catechol, 3,4-dihydroxycinnamic acid and noradrenaline indicated that although rates of reaction may vary, (noradrenaline was found to be more stable than adrenaline), the free energies of activation ( $\Delta G^*$  values, Section 1.3) were almost identical (28). They inferred from this that the nature of the reaction in each case is the same but side-chain variations cause differences in solvation, protonation etc., which influence the rate of reaction.

Studies on phenylephrine by El-Shibini, Daabis and Motawi led them to the conclusion that this drug can also cyclise in a similar manner to adrenaline in alkaline solution (39) (Fig. 3).

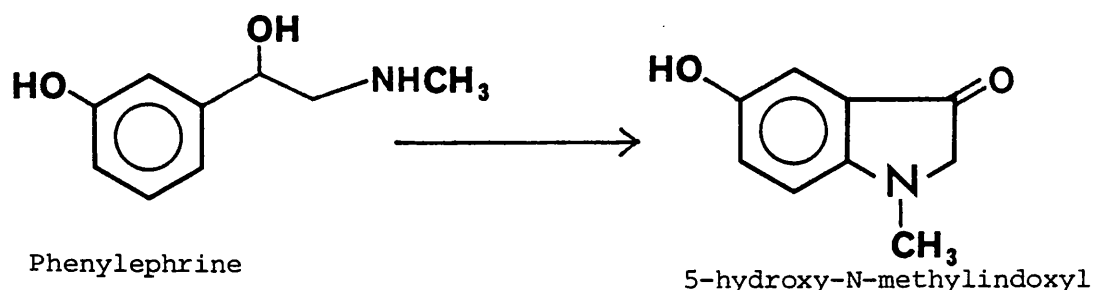


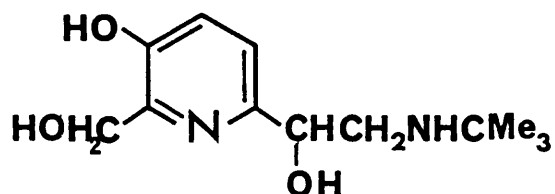
Fig. 3



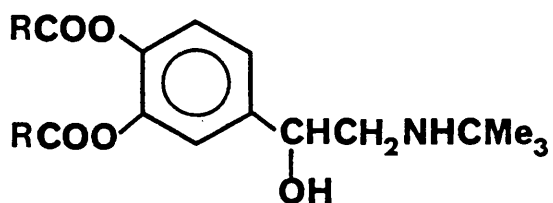
Under acidic conditions, degradation was found to be too slow for convenient study. Further investigations (40) demonstrated that copper has a strong catalytic effect on the reaction but that iron has virtually no effect. Unfortunately they did not examine iron together with EDTA to confirm a parallel with the results of Green, Mazur and Shorr (38), although they did show that EDTA prevents the catalytic action of copper. The reduced rate of breakdown of phenylephrine relative to adrenaline is due to the loss of one phenolic hydroxyl from the benzene ring and reflects the lower reactivity of phenol compared to that of catechol.

Although the synthetic sympathomimetic drugs have been widely available for many years, very little data on their stability has been published. Salbutamol in particular has been the subject of only one published stability study which was of limited scope and used fluorimetric and colorimetric assays (41). The work was designed to determine the stability of salbutamol solutions under conditions of hospital use, particularly in relation to intravenous infusion therapy. Phosphate buffers over the pH range 6.9 to 8.3 were used, as well as 5% dextrose solution, a common vehicle for intravenous medication. Solutions under investigation were sealed in over-sized ampoules and stored at temperatures ranging from 40° to 70°. Pseudo-first order rate constants were calculated although in many cases the graphs showed marked deviations from linearity. Arrhenius plots were found to be non-linear and the authors inferred that there was a change in reaction mechanism above 60°. They concluded that salbutamol was considerably more stable in dextrose

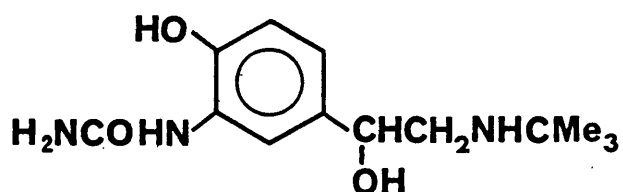
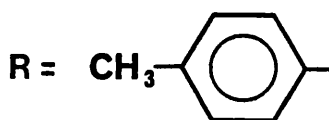
solutions than in solutions of near neutral pH and that salbutamol solutions should not be heat sterilised. Related compounds which have been examined are pirbuterol (42), bitolterol (43) and carbuterol (44).



Pirbuterol



Bitolterol



Carbuterol

Fig. 4.

The bitolterol study was concerned purely with the rate of hydrolysis of the ester linkages and it was found that reduction of the dielectric constant with non-aqueous solvents considerably increased the shelf-life of the solution (43). Carbuterol differs from the catecholamines by degrading significantly under anaerobic conditions (44). The main objectives of the published investigation were to define the kinetics of the breakdown under such conditions and to identify

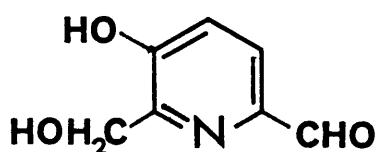
breakdown products. The major degradation product was isolated and characterised by mass spectrometry as the internal, cyclic carbamate:



Cyclic carbamate

Pseudo 1st order kinetics were followed, with catalysis by phosphate buffers.

Pirbuterol, the compound most closely related to salbutamol, was found to follow 1st order kinetics during its breakdown at pH 7 and 90° (42) and the major breakdown product was identified as 2-hydroxymethyl-3-hydroxy-6-formylpyridine. A full



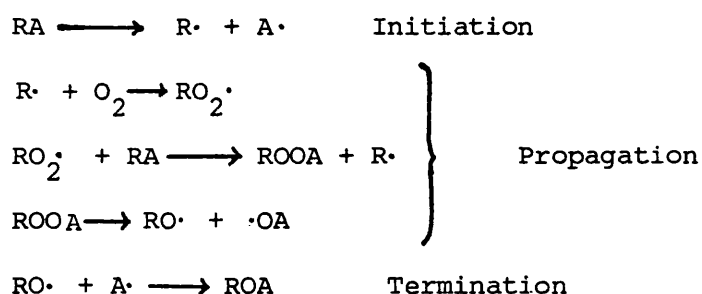
2-hydroxymethyl-3-hydroxy-6-formylpyridine

pH-stability profile was produced (pH 1-14), the drug showing a point of minimum stability at pH 8 but no pronounced point of maximum stability. Unlike other studies, the data was obtained without the use of buffers, the pH of the test solution being periodically adjusted by the addition of acid or alkali.

## 1.5 Oxidation

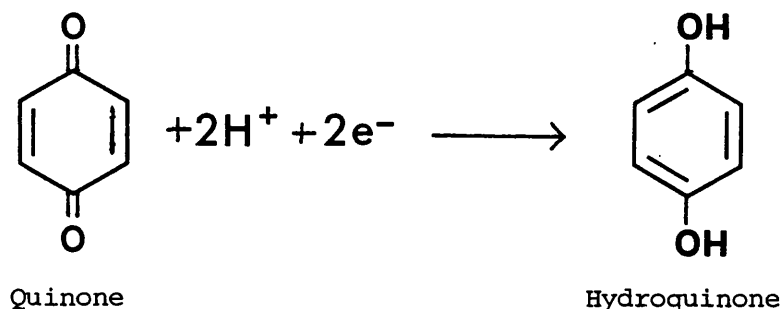
Since oxidation plays an important role in the degradation of catecholamines and related compounds (Section 1.4) it is important to consider the factors which affect this particular type of reaction.

Oxidation is defined as loss of electrons and so molecular oxygen need not be involved at all. However, in many pharmaceutical systems, oxygen is readily available and becomes incorporated into the reaction mechanism. These reactions are often mediated by free radicals, produced by the action of heat or light and are therefore self-propagating. A typical chain reaction may be demonstrated by considering a drug, RA



The free radical nature of these reactions results in complex kinetics and the overall process has a non-integral order (Section 1.3). The reactions are often catalysed by heavy metal ions which can increase the rate of free radical production.

Other oxidations are catalysed by hydrogen or hydroxyl ions. The hydroquinone-quinone reaction is a typical example of this type of redox system and plays an important role in the degradation of adrenaline (Section 1.4). Quinones are usually coloured compounds whilst hydroquinones are colourless and so oxidation is easily detected by the development of colour in the solution.



It may be predicted from the redox equation that oxidation will be minimised in acid solutions. This is often true in the case of pure solutions, but buffering to the required pH does not always solve the problem, since the buffer salts themselves can act as catalysts or stabilisers. Boric acid, for example, has been shown to stabilise catecholamine solutions (45-48) by chelating with the phenolic groups, whilst iron, at acidic pH can catalyse the oxidation (49). Such effects, coupled with the fact that many drug molecules have several functional groups which could be oxidation-sensitive, can result in complex pH-stability profiles (42,50).

Temperature effects for oxygen dependent processes tend to be unpredictable because of the alteration in oxygen solubility. Normally, a rise in temperature causes an increase in reaction rate but the accompanying fall in oxygen solubility could decrease the rate of reaction.

## 1.6 Sugar Chemistry

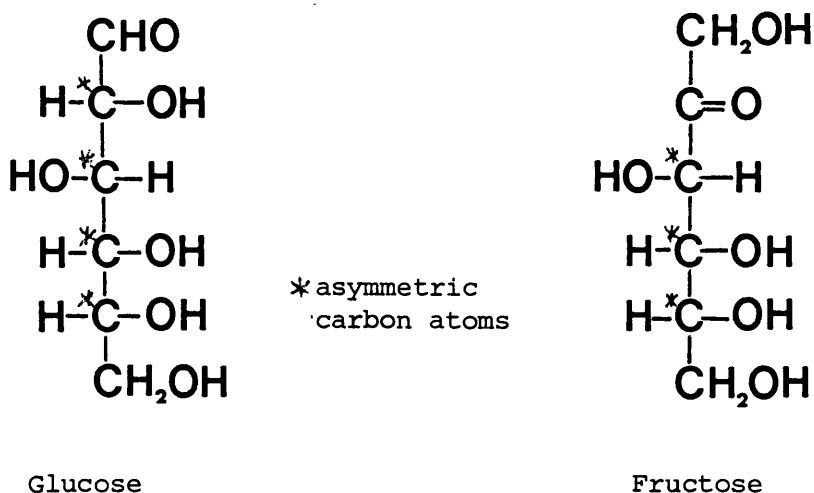
Pharmaceutical syrups contain large quantities of sugar which not only sweeten the preparation but also increase its viscosity, giving the liquid "body". Because they frequently represent the

major ingredient of such preparations, the properties of the sugars may have a profound effect on the properties and stability of the whole formulation.

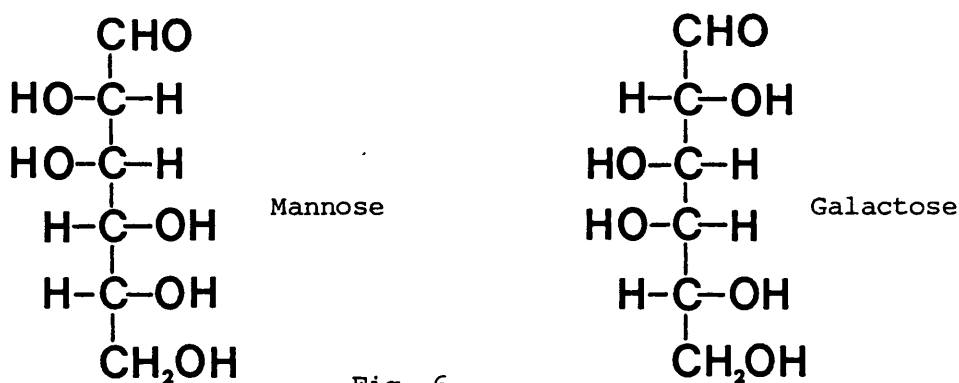
Sugars are members of the class of compounds known as carbohydrates. The carbohydrates are commonly sub-divided into sugars, oligosaccharides and polysaccharides although the boundaries between them are indistinct. Sugars include the mono- and disaccharides. Oligosaccharides generally contain up to ten monosaccharide units and polysaccharides are the high molecular weight carbohydrates containing hundreds or even thousands of monosaccharide units.

Most monosaccharides, particularly the common ones, contain five or six carbon atoms although some may have as few as three and others as many as seven. Many monosaccharides occur naturally as metabolic products of living organisms. In general, they have a sweet taste and are soluble in water, making them popular as flavouring agents in food and drinks as well as pharmaceuticals.

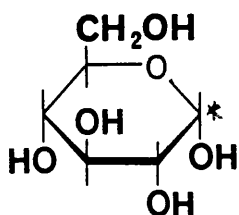
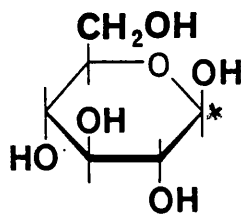
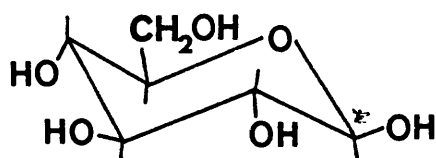
The commonest sugars are polyhydroxy aldehydes or ketones and are optically active, due to the presence of several asymmetric carbon atoms (Fig. 5). Glucose and fructose are examples of an aldohexose and ketohexose respectively. The "hexose" portion of the name refers to the number of carbon atoms in the molecule (six in this case), and the keto- or aldo- prefix defines the nature of the carbonyl group. It can be seen

Fig. 5

that both these sugars can have several isomers which vary in the configuration of the hydroxyl group at each asymmetric centre. These isomers represent different chemical entities and include the sugars galactose and mannose amongst others (Fig. 6).

Fig. 6

The sugars in Figs. 5 and 6 have been drawn as straight-chain compounds since this makes it easier to identify the asymmetric centres and to appreciate the relationship the isomers bear to each other. In fact, the molecules usually exist as cyclic structures by forming internal hemiacetals with an additional asymmetric centre (51). The most common way of representing this is by the Haworth formula as shown in Fig. 7. This is only a diagrammatic representation since the rings are not really planar but are buckled into a "chair" conformation.

 $\alpha$ -D-glucopyranose $\beta$ -D-glucopyranose $\beta$ -D-glucopyranose (chair form)

\* new asymmetric carbon atom

Fig. 7

Some sugars, such as fructose, form five-membered rings and are therefore termed furanose instead of pyranose which defines the ring as six-membered. The new asymmetric carbon atom is called the anomeric centre and the  $\alpha$  and  $\beta$  forms are known as anomers.

In aqueous solutions the three forms of the sugar ( $\alpha$  and  $\beta$  rings and the acyclic structure) exist in dynamic equilibrium with each other. The percentage in the open form at any time is less than 0.5% (52) but the addition of a reagent which displaces the equilibrium by reacting purely with the aldehyde form, causes rapid and complete conversion to this structure (53).

The most reactive site in sugar molecules is the carbonyl



carbon atom; the hydroxyl groups can be methylated or acetylated relatively easily, but most important reactions occur at the carbonyl group. Isomerisation can take place easily at this point, particularly in neutral or basic solutions, via an enediol structure. This is known as the Lobry de Bruyn-van Ekenstein rearrangement (Fig. 8).

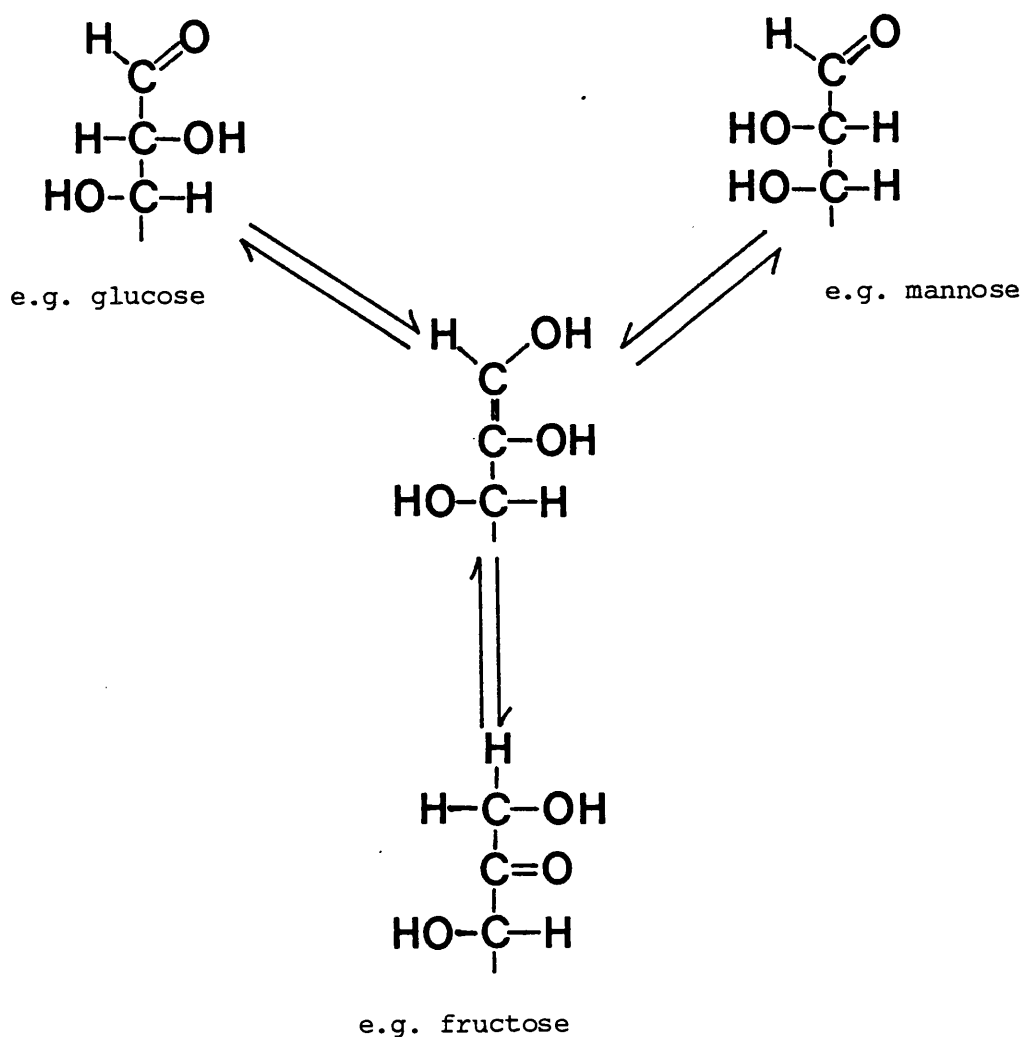
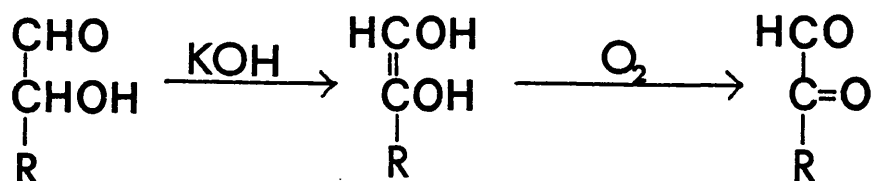


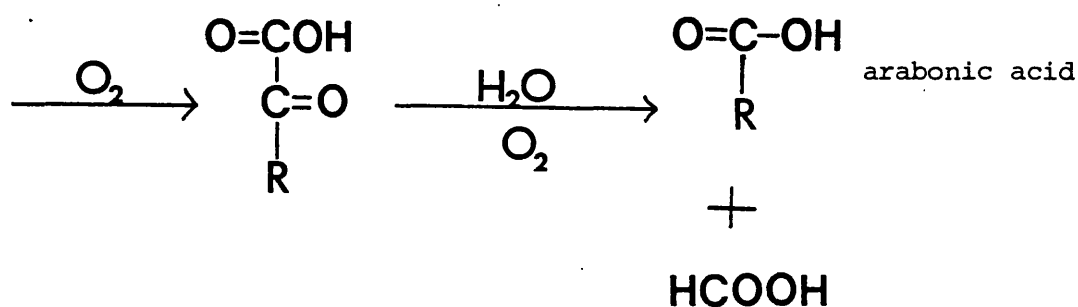
Fig. 8

This isomerisation is the cause of the formation of mixed products in many reactions involving sugars and so, for improved yields, it is often recommended that reactions be performed under acidic conditions, where possible. In the case of glucose, the rearrangement leads to the generation of fructose and mannose.

In neutral or alkaline solution, sugars are readily oxidised by molecular oxygen with loss of formate. This produces an acid with one carbon less than the original sugar e.g.



glucose



70% yields of arabonic acid have been obtained from glucose by carrying out the reaction in 1N potassium hydroxide solution (54) and the method has been used to prepare other aldonic acids from the relevant sugars (55).

In acidic solutions the monosaccharides are much more stable, most of them showing maximum stability around pH 3 to 4 (56-58). Small amounts of disaccharides may be formed by dehydration between two monosaccharide units but the reaction is seldom of any practical significance. Stronger acids cause intramolecular dehydration with the formation of furan derivatives (Fig. 9). This is particularly common with keto sugars.

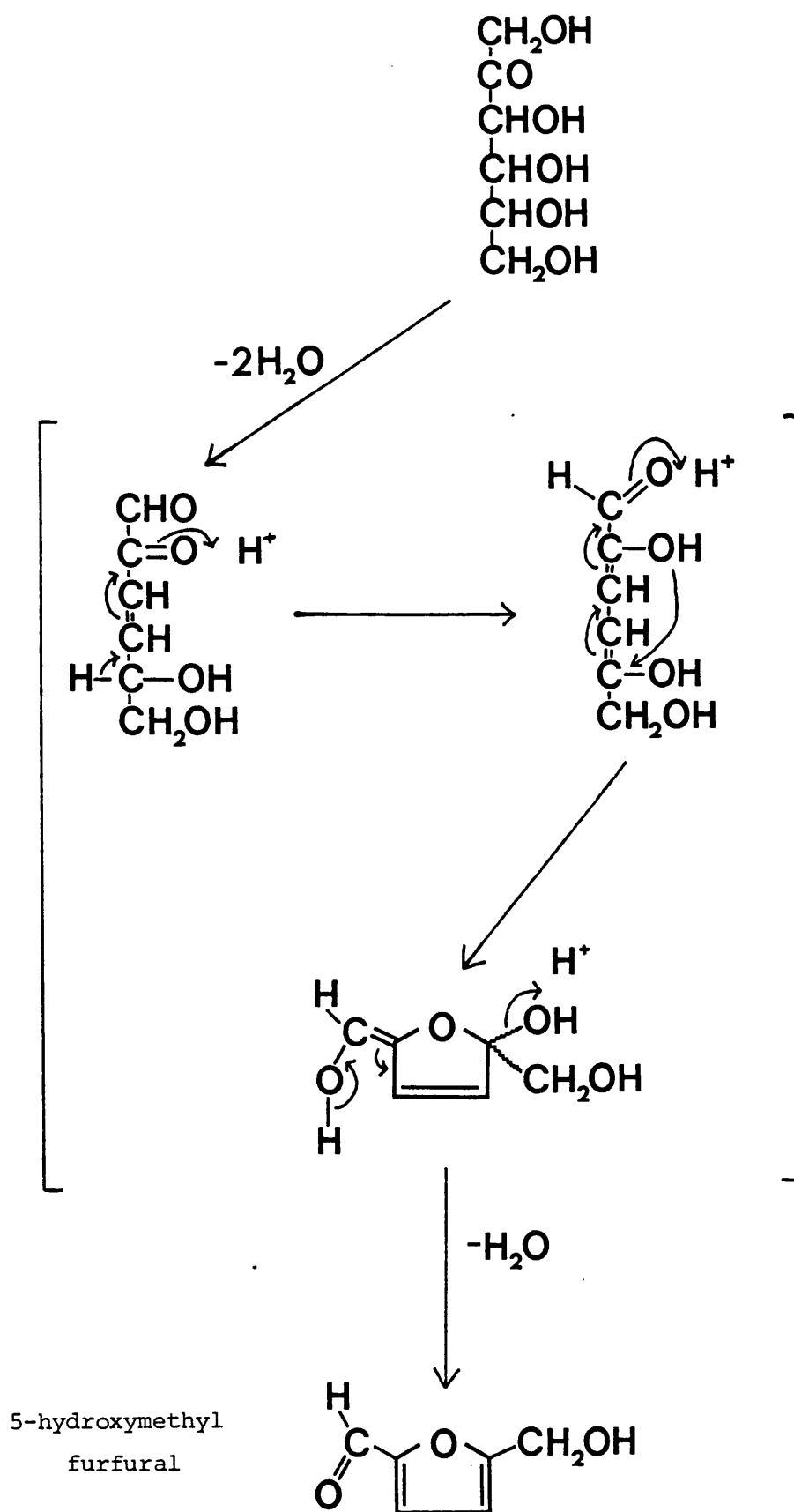


Fig. 9

The product of the reaction depicted in Fig. 9, 5-hydroxymethylfurfural, has been implicated in many sugar reactions, particularly those involving the breakdown and discolouration of sugar solutions (58, 59).

Other reactions at the carbonyl carbon include glycoside formation with alcohols and glycosylamine formation with amines (60, 61) as shown in Fig. 10.

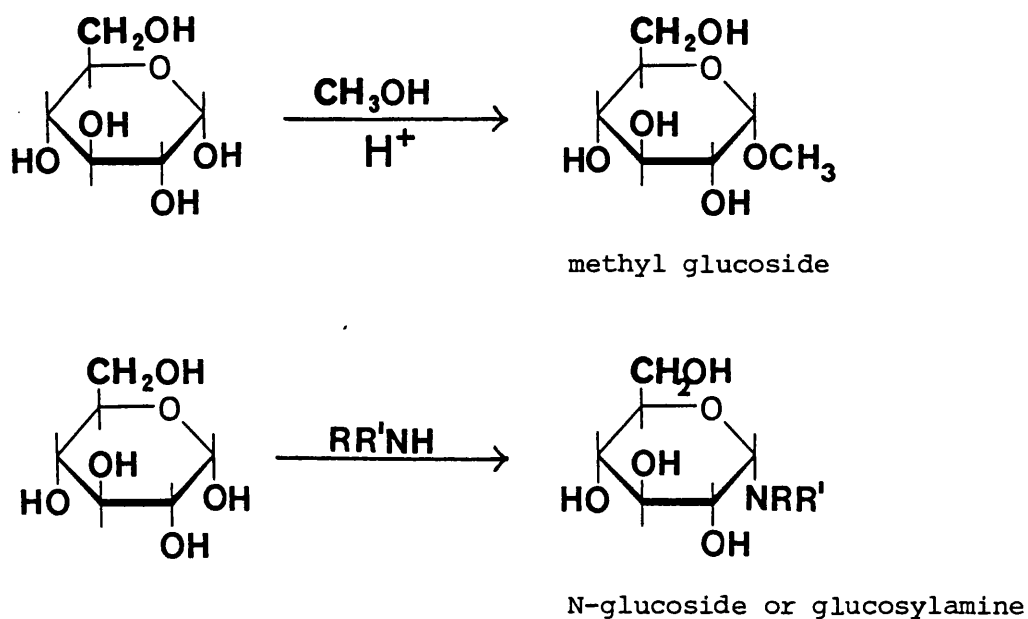
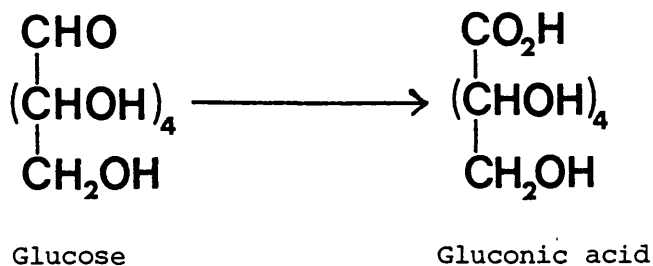


Fig. 10

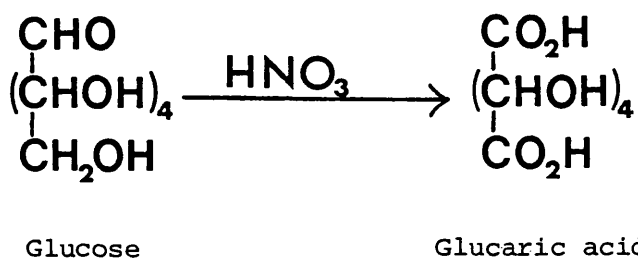
No acid catalyst is needed for the amine reaction, unlike the methylation, and it has been suggested (60) that the acyclic form of the sugar is involved. Both glycosides and glycosylamines are hydrolysed back to the free sugars by aqueous acids (60, 61).

The carbonyl group is readily oxidised by a variety of

oxidising agents, in addition to the oxidation in alkaline solution previously discussed. Mild oxidation with bromine water yields the corresponding aldonic acid.

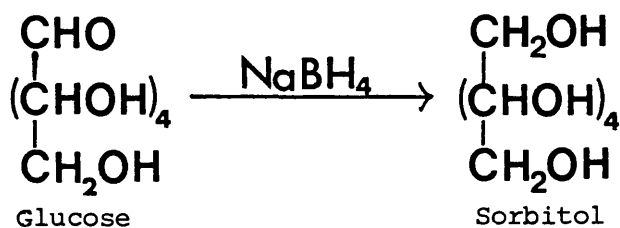


More powerful reagents, e.g. nitric acid, attack both ends of the molecule producing an aldaric acid.



Reduction of the carbonyl yields a polyhydroxy alcohol or alditol.

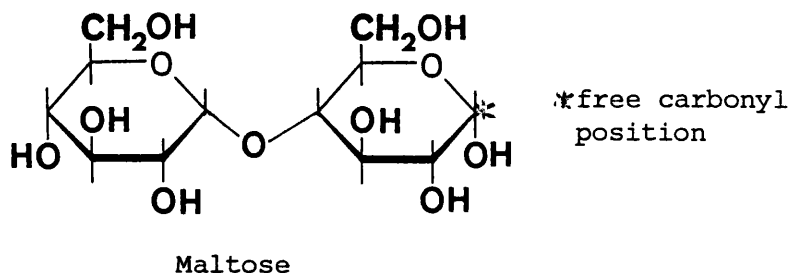
Glucose may be reduced to sorbitol using sodium amalgam or sodium borohydride.



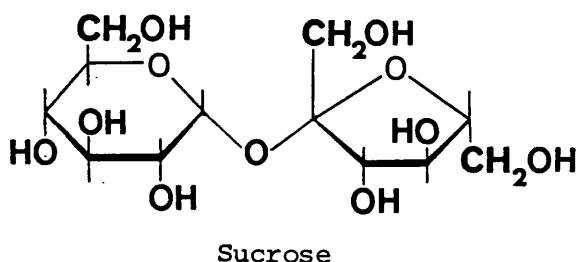
Alditols are more stable than their corresponding sugars since they lack the reactive carbonyl. They are also unable to form cyclic structures in solution. They can be oxidised to sugars by hydrogen peroxide in the presence of ferrous salts but are unaffected by dilute acids and alkalis.

Disaccharides are formed by loss of water between two monosaccharide units. This reaction involves the anomeric

centre in at least one of the units but the other monosaccharide may retain a free carbonyl group and therefore its reducing properties. Maltose is an example of such a sugar, being composed of two glucose units joined through the 1,4 positions:



Sucrose, on the other hand is a non-reducing sugar, because both monosaccharide units are joined through their anomeric centres.



Unlike maltose, a sucrose molecule contains two different monosaccharide residues. It is, in fact, a glucosyl fructose and may be hydrolysed with dilute acid to yield equal quantities of glucose and fructose. This hydrolysis results in a change in optical rotation of the solution from positive to negative and the process is therefore often called inversion. The reason for the change is that fructose is more strongly laevorotatory than glucose is dextrorotatory. The chemical properties of the disaccharides are determined largely by their constituent monosaccharide units but reactions involving the carbonyl group are impossible in sugars such as sucrose without prior hydrolysis.

Polysaccharides include substances such as starch and

cellulose which are naturally occurring plant storage and structural polysaccharides respectively. Like the di- and oligo-saccharides, they can be hydrolysed to their component units by dilute acids. Specific enzymes exist which can selectively hydrolyse certain linkages whilst leaving others intact and use of these has proved of great value in structural determinations on these complex molecules.

Semi-synthetic cellulose derivatives have been prepared from purified natural cellulose by the use of various reagents. The objective is to modify the properties of the compound by replacing some of the hydroxyl groups with, for example, methoxy or acetyl groupings. The modified celluloses are used quite commonly in pharmacy for a variety of purposes. Many tablet film coatings consist of cellulose derivatives such as hydroxypropylmethylcellulose. Methylcellulose and carboxymethylcellulose are used as thickening agents and laxatives (62).

Cellulose itself is not soluble in water but the semi-synthetic derivatives are slowly soluble, forming viscous liquids or gels. They are available in different grades with fairly well defined properties so that a suitable product for a given purpose can be readily selected.

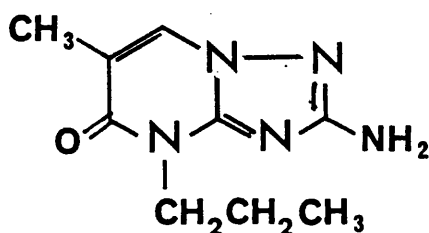
#### 1.7 Interactions Between Drugs and Syrup Excipients

By far the major component of pharmaceutical syrups is sugar of some kind. Glucose and sucrose are probably the most popular but sorbitol is also used quite widely (63). Other important ingredients include preservatives and antioxidants

together with flavourings, colourings and buffer salts.

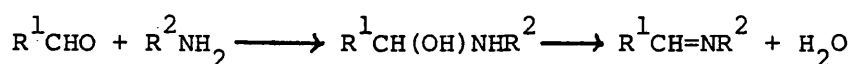
Although it is now well known that buffer salts and stabilisers such as EDTA can affect drug stability (Sections 1.4 and 1.5) sugars and carbohydrates, when used in pharmaceutical preparations, are usually regarded as inert and their interactions with drugs have seldom been investigated. Penicillins have received some attention because they are frequently added to intravenous infusions containing glucose, and it has been shown that glucose and fructose accelerate their breakdown at neutral and alkaline pH (64 - 68). With reference to oral formulations, sucrose has been found to affect penicillin inactivation, again in neutral or alkaline solutions (64, 69). The mechanism of the sucrose-benzylpenicillin reaction has been investigated by Bundgaard and Larsen (13) and they showed that an ester is formed between the penicillin and sucrose. This ester then hydrolyses to produce benzylpenicilloic acid. The rate of ester formation and hydrolysis was considerably greater than the rate of direct penicillin hydrolysis to benzylpenicilloic acid and so a catalytic effect was observed.

Kitson et al. (16) have investigated the effect of sugars on the stability of the drug 2-amino-6-methyl-5-oxo-4-n-propyl-4,5-dihydro-1,2,4-triazolo [1,5-a] pyrimidine. Both reducing



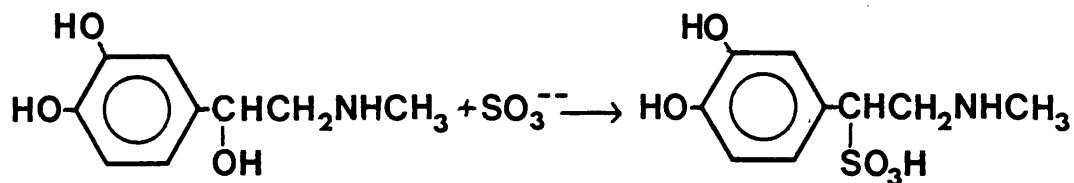


and non-reducing sugars were examined and the former were found to result in the least stable systems. However, in the presence of oxygen, terminal alcohol groups of non-reducing sugars were believed to be oxidised to aldehydes, allowing reaction to take place as before. The mechanism they proposed involved the formation of Schiff bases by reaction between the amino group of the drug and the aldehyde of the sugars.

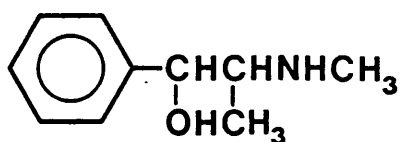


Later work by the same authors (17) revealed that methanol and acetone did not react but acetaldehyde caused considerable loss of drug. This provided some confirmation for the proposed mechanism. However acetone would also have been expected to react, as sorbose, a keto sugar did. This anomaly can be explained if the keto sugars undergo the Lobry de Bruyn-van Ekenstein rearrangement under the alkaline conditions of the experiment, to yield the corresponding aldo sugars which are able to react by the proposed mechanism.

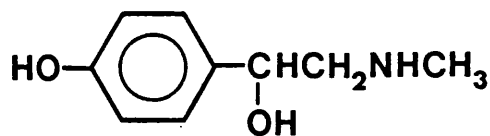
Other pharmaceutical excipients have been found to interact with drug molecules. A prime example is metabisulphite which is often used as an antioxidant to stabilise oxygen-sensitive formulations. Adrenaline is a particularly oxidation-prone drug (Section 1.4) and anything which removes oxygen from the formulation would be expected to increase its stability. Unfortunately, adrenaline reacts specifically with metabisulphite and the reaction has been studied by Schroeter and others (14, 15, 47, 48, 70). The mechanism they proposed involves nucleophilic attack by the sulphite ion as shown overleaf.



Ephedrine and other compounds which lack phenolic hydroxyls were found not to react, although synephrine did react.



Ephedrine



Synephrine

Phenolic hydroxyls in the meta position failed to activate the molecule to the reaction and compounds without a hydroxyl on the  $\alpha$ -carbon atom were also unreactive.

Some Japanese workers have recently patented a method of avoiding this problem (71). They stabilised ampoules of adrenaline injection by adding 4% reducing sugars to the solution. Glucose, galactose, fructose, maltose and lactose were all tested and it is claimed that after heating the ampoules, the stabilised injections retained more than 87% adrenaline whilst a control had none at all. This suggests that the limited quantity of oxygen available in the sealed ampoules, preferentially oxidises the sugars rather than the adrenaline, a useful, if somewhat surprising, result. Ascorbic acid has also been considered as an alternative antioxidant(72) but was found to produce hydrogen peroxide, a powerful oxidising agent.

### 1.8 Assay of Salbutamol Sulphate

Several assay methods have been used for salbutamol, its salts and related compounds (Table 1). However, most suffer from major disadvantages, when applied to kinetic studies in the presence of large quantities of breakdown products.

Table 1. Assay techniques used in stability studies on salbutamol and related compounds.

| Drug           | Reference | Assay Method(s)                                    |
|----------------|-----------|--|
| Salbutamol     | 41        | Fluorimetry and Colorimetric Reaction              |
| Carbuterol     | 44        | HPLC (Cationic exchange) and Colorimetric reaction |
| Pirbuterol     | 42        | TLC  |
| Adrenaline     | 37        | Spectrophotometry of derivative                    |
| Terbutaline    | 74        | GLC and TLC  |
| Catecholamines | 28        | Fluorimetry  |
| Bitolterol     | 43        | TLC and HPLC                                       |
| Phenylephrine  | 39        | Colorimetric reaction                              |
| Phenylephrine  | 75        | TLC and GC   |

Fluorimetry and uv spectrophotometry are the two simplest techniques. However, they are suitable only for pure solutions of the drug, since many possible degradation products could have similar emission or absorption spectra. Salbutamol has a  $\lambda_{\text{max}}$  at 278 nm but this is primarily an aromatic absorption characteristic of a substituted benzene ring, and so any similar aromatic molecule will interfere with the assay. A similar problem

arises with fluorimetry although it has been used by some workers for stability studies (41).

A colorimetric assay based on the formation of an indoaniline dye is used in the BPC 1973 for salbutamol inhalers (73). The product under examination can be assumed to have undergone only a small amount of decomposition and the assay is intended mainly to ensure that the metering device delivers the correct dose. It is therefore unnecessary to have a highly specific, stability indicating assay. The main problem with this method is that half the salbutamol molecule is "lost" during the reaction (Fig. 11) and so breakdown products with modifications in this portion of the molecule (the amino side-chain), will be assayed as salbutamol. The method is therefore of little value in stability and kinetic studies, although it was used during the developmental work on salbutamol.

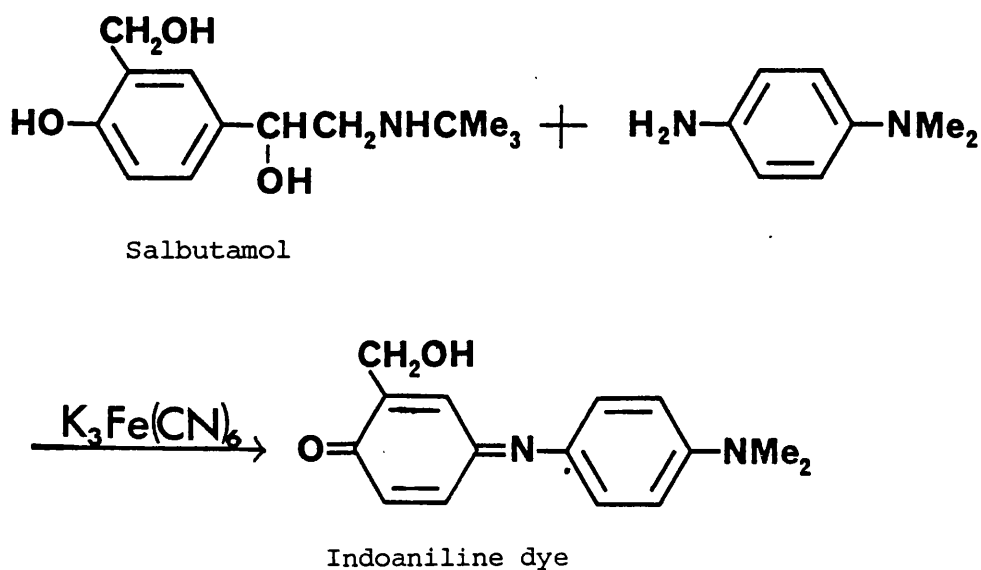


Fig. 11

A similar colorimetric reaction with 4-aminophenazone has been used by some workers to assay salbutamol in the presence of propylene glycol, sodium metabisulphite and calcium disodium edathamil (78).

Gas chromatography has been used in metabolic and biochemical studies for the measurement of drug levels in body fluids (76). This technique requires the drug to be derivatised, a comparable procedure to that used in a colorimetric reaction, and subject to similar potential drawbacks. Detection and measurement of the chromatographic peaks used mass fragmentography, an expensive and difficult method for routine assay work. Mass fragmentography has also been used alone for biochemical studies but careful extraction procedures are then necessary to separate endogenous compounds (76, 77).

Thin layer chromatography (TLC) is a highly versatile and sensitive technique which has been applied to most drugs, including salbutamol. It is used as a quality assurance check on the drug in the B.P. (79) but is not readily adapted to accurate quantitative work.

The only remaining technique of any relevance is high performance liquid chromatography (HPLC) and this was selected as the most promising method of assaying salbutamol accurately in the presence of its degradation products. HPLC has the potential to combine the resolution efficiency of TLC with easy quantification and so it is not surprising that HPLC separations have been developed for very many drugs, both in biological fluids

and in pharmaceutical formulations. The variety of column packing materials now available has expanded the versatility of the technique tremendously and should enable any separation problem to be solved, given enough time, money and patience!

### 1.9 HPLC Theory

There are three principal types of HPLC, adsorption, ion exchange and reversed phase. They vary in the nature of the column packing material but all rely on the same general principles. Separation is achieved by injecting a small sample of the drug solution onto a packed column of the stationary phase and eluting the various components of the sample by pumping a suitable liquid mobile phase through the column. The stationary phase may be silica, a synthetic polymer or a modified silica which has different functional groups bonded to the silica backbone. The mobile phase is largely dependent on the stationary phase but is usually an organic-aqueous mixture containing buffer salts or surfactants. The main difference between HPLC and traditional column chromatography lies in the particle size of the column packing material. HPLC uses materials with a particle size of 5 - 10  $\mu\text{m}$ . This means that the columns are much smaller (4mm I.D.) and shorter (10 - 30 cm) than traditional chromatography columns but because the small particles present a high resistance to flow, the mobile phase has to be pumped through under pressure.

Separation of the components of the mixture is dependent on their different relative affinities for the stationary and mobile phases. Compounds which are strongly adsorbed by the

packing material will have long retention times, whilst those which are readily soluble in the mobile phase will elute rapidly. The precise mechanism of separation varies with the packing material. Polar materials such as silica rely mainly on adsorption, polar molecules having a high affinity for the exposed hydroxyl groups of the silica. Mobile phases used with silica columns generally contain a high proportion of organic solvent, sometimes with the addition of acids or bases to ensure that the solutes are in the desired ionisation state. This chromatographic mode is most useful for compounds of medium polarity. Highly polar molecules tend to be adsorbed so strongly that they are difficult to elute and hydrophobic compounds are insufficiently retained to enable separation to take place.

Partition plays a major role in systems where a stationary liquid phase is used. This is achieved by impregnating an "inert" solid packing material with the chosen liquid phase and is an extension of the technique of liquid-liquid extraction.

Ion exchange HPLC works by standard ion-exchange mechanisms, cationic exchangers being used to separate cations and anionics for anions. Both strong and weak exchangers are available and aqueous buffer solutions are the most usual mobile phases.

Reversed-phase packing materials are a relatively recent development but have rapidly become established as highly versatile analytical tools. They are available as chemically modified silicas, in which the exposed silanol groups have been alkylated. The most common modification is the octadecyl ( $C_{18}$ )

group which has the effect of rendering the silica hydrophobic instead of hydrophilic. As a result, non-polar molecules are adsorbed by the material and such columns originally found application in the separation of hydrocarbons. However, it was soon discovered that the addition of certain ions to the mobile phase enabled good separations of polar solutes to be achieved, due to ion-pair formation.

Although most ionic molecules separate into their constituent ions in aqueous solution, some large ions are more stable when paired with a counter-ion, enabling partition into a non-aqueous environment. Such a mechanism is the basis of many extraction procedures used, for example, in the extraction of drugs from biological samples. The counter-ions used in HPLC are often surfactants such as sodium dodecyl sulphate or cetrимide and so the term "soap chromatography" (80) is sometimes applied to the technique. The precise mechanism of the separation is not yet clear (81). The ion-pair may form in the mobile phase and then be adsorbed by the stationary phase (82, 83). Alternatively, the long-chain counter-ion may be adsorbed first, enabling a type of ion-exchange process to take place, as proposed by Knox and coworkers (80, 84). Recently Bidlingmeyer *et al.* have proposed a new mechanism which seems to be a mixture of the two (85).

The efficiency of the column can be described mathematically in the same way, regardless of the actual physico-chemical interactions involved. Several parameters are necessary in order to be able to compare the efficiency of different columns



and to check on the continuing integrity of an established column.

The first important parameter, which is widely used, is the column capacity ratio,  $k'$ . Its main use is as a retention parameter. Each solute in a mixture will have an individual  $k'$  value and the greater the difference in  $k'$  between two components, the greater will be their separation.

$$k' = \frac{n_s}{n_m}$$

$n_s$  = number of moles of solute in stationary phase

$n_m$  = number of moles of solute in mobile phase

In practice,  $n_s$  and  $n_m$  cannot be measured and a more useful definition of  $k'$  relates it to retention times which are readily determined from the chromatogram.

$$k' = \frac{t_r - t_o}{t_o} \quad \dots (10)$$

$t_r$  = solute retention time

$t_o$  = retention time of unretained solute

Retention times tend to vary between chromatographic systems due mainly to differences in void volumes and packing efficiency. Use of capacity ratios eliminates this problem and enables results from different laboratories or different pieces of equipment to be compared. The ratio of  $k'$  values for two peaks in the chromatogram is known as the separation factor,  $\alpha$

$$\alpha = \frac{k'_a}{k'_b}$$

The other major parameter used to define a chromatographic system is  $N$ , the number of theoretical plates in the column. The greater

the value of  $N$ , the more efficient the column.

$$N = 16 \left( \frac{t_r}{t_w} \right)^2 = \left( \frac{t_r}{\sigma} \right)^2 \quad \dots (11)$$

$t_w$  = peak width in time units

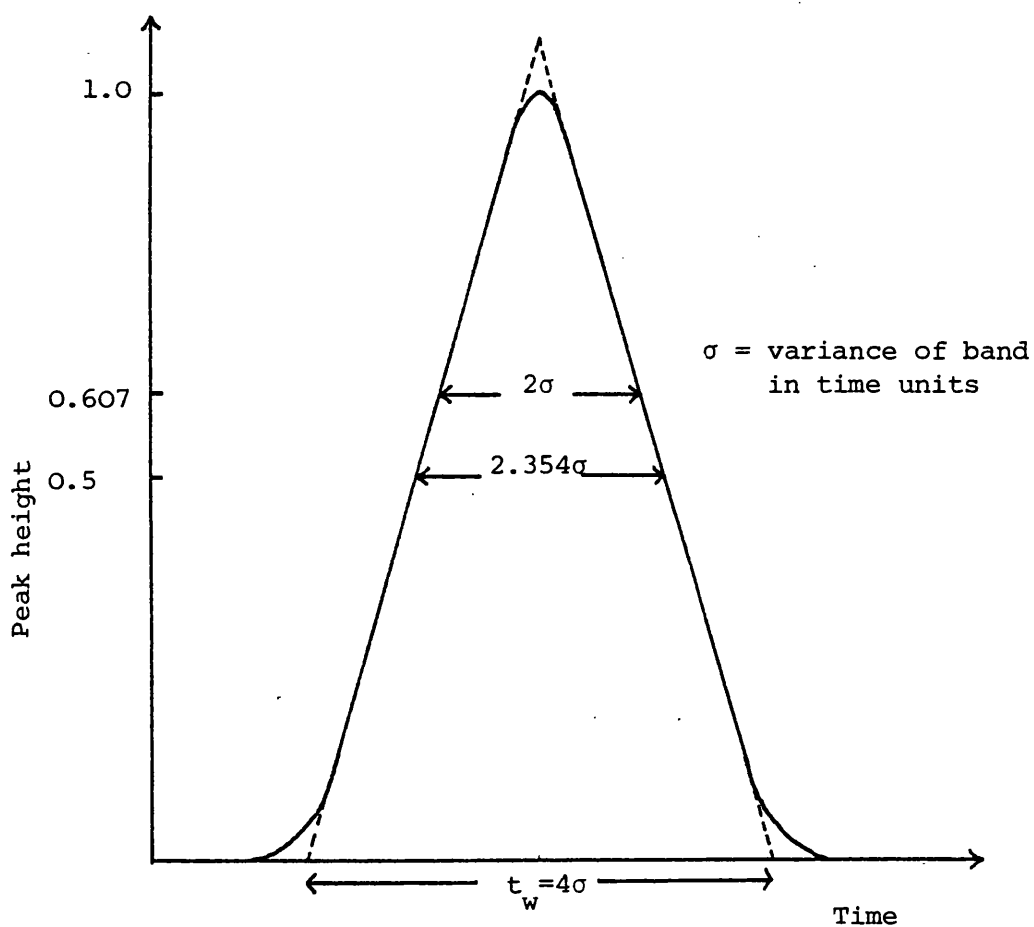


Fig. 12

An alternative way of describing column efficiency is to calculate the height equivalent to a theoretical plate (HETP). The smaller this value, the greater the separating power of the column

$$H = \frac{L}{N}$$

$H$  = HETP

$L$  = length of column

The concept of theoretical plates developed from an analogy with distillation columns (86, 87). A number of equilibria are considered to arise within the chromatographic column, each one comparable to the equilibrium which exists on a plate in a distillation column. Each "plate" represents a progressive increase in the resolution of the components, and so the more plates present in the column, the greater will be its efficiency. A typical 10 cm column should contain around 4000 to 5000 plates.

A column with a low plate number may separate the components of the mixture but with such broadening of the bands as to make the chromatogram useless for analytical purposes. Ideally, peaks should be as narrow as possible with baseline resolution between successive peaks but in practice this is not always possible. A compromise must therefore be reached between ideal separation and practical analysis time. The resolution,  $R_s$  is defined as follows:

$$R_s = 2 \left( \frac{t_{R_1} - t_{R_2}}{t_{w_1} + t_{w_2}} \right)$$

$t_R$  = retention time of solutes 1 and 2

$t_w$  = peak width of solutes 1 and 2

When  $R_s = 1.5$  the overlap of two adjacent peaks is less than 1% but even at  $R_s = 1.0$ , resolution is sufficient to make the two peaks readily recognisable, even if one is much smaller than the other (Fig. 13).

Analysis times need to be kept as short as possible for two main reasons. The obvious one is that more samples can be processed

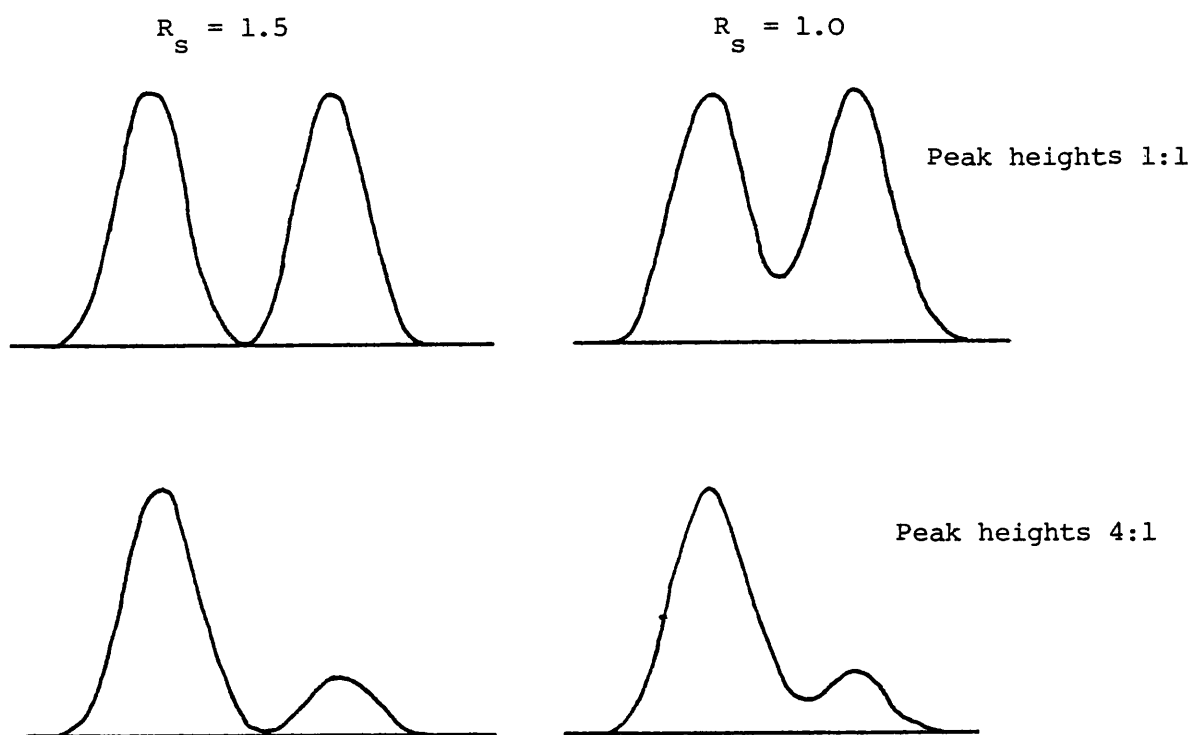


Fig. 13

in a given time and this is a great advantage in routine assay work with large numbers of samples. The other reason is that band broadening by solute diffusion is reduced if the analysis time is short. Solute diffusion is the cause of the progressive broadening of chromatographic peaks with time, resulting in sharp, narrow early peaks, but broad less distinct peaks for solutes with long retention times. Since it is difficult to accurately measure such peaks, HPLC systems tend to be developed so that the most important peaks elute early in the chromatogram.

Gradient elution provides a means of avoiding this problem with strongly retained solutes. In this case the composition of the mobile phase varies with time so that its elution power steadily increases. In this way a strongly retained compound

can be eluted much more quickly than in isocratic HPLC where the mobile phase composition remains constant. The disadvantage is that the column has to be re-equilibrated to the starting composition before the next sample can be injected and so the overall analysis time may not be any shorter, although the chromatography may be considerably improved.

## Section 2.

EXPERIMENTAL METHODSA. ANALYTICAL METHODSMaterials

Salbutamol sulphate was B.P. grade and was used as supplied by Allen and Hanburys Research Ltd. (now Glaxo Group Research), without further purification.

1-(4-hydroxy-3-methylphenyl)-2-(t-butylamino)ethanol (AH 4045) was used as supplied by Allen and Hanburys Research Ltd., without further purification.

Acetonitrile and methanol were HPLC grade (Rathburn Chemicals Ltd.)

Isopropanol and ethyl acetate were AnalaR grade (BDH Chemicals Ltd.)

Sodium dodecyl sulphate was a "specially pure" grade (BDH Chemicals Ltd.)

Perchloric acid was AnalaR grade (BDH Chemicals)

SP-Sephadex C25 was obtained from Pharmacia Ltd.

Ammonium chloride was AnalaR grade (BDH Chemicals Ltd.)

All other chemicals, solvents and reagents were of AnalaR quality wherever possible.

Water was distilled in an all glass still.

Equipment

HPLC System: Pye Unicam LC3 chromatograph

Pye Unicam LC3UV variable wavelength detector

Pye Unicam Minigrator DP88 computing integrator

Tekman TE200 flat bed chart recorder

10 cm stainless steel column (0.4 cm I.D.)

packed with Spherisorb S5 ODS

Rheodyne 10  $\mu$ l loop valve

Pye Unicam SP 1800 scanning spectrophotometer

Pye Unicam SP 500 spectrophotometer

Büchi Rotavapor-R rotary evaporator

Jeol FX90Q nuclear magnetic resonance spectrometer

Jeol JNM-PS-100 nuclear magnetic resonance spectrometer

AEI MS12 mass spectrometer

Analytical glassware was mainly Grade A and was cleaned regularly with "chromic acid".

TLC plates were precoated and obtained from Anachem Ltd..

The adsorbent was silica gel G with a  $uv_{254}$  fluorescent indicator.

Two thicknesses were used, 250  $\mu$ m and 1000  $\mu$ m (for preparative work).

The chromatography columns for the Sephadex-extraction of analytical samples consisted of glass tubing 60 cm x 0.6 cm I.D. fitted with a Teflon burette tap at the base.

## 2.1 Development of HPLC Assay

Almost every possible mode of HPLC has been used for the separation of catecholamines and phenethylamines as demonstrated in Table 2.

| HPLC Mode         | Packing Material               | Compounds Separated                                 | Ref. |
|-------------------|--------------------------------|---|------|
| Adsorption        | Partisil                       | Phenethylamines                                     | 88   |
|                   | Spherisorb A20                 | Catecholamines                                      | 84   |
|                   | Corasil II                     | Amphetamines  | 89   |
| Anionic exchange  | DAX4                           | Amphetamines  | 89   |
| Cationic exchange | Zipax SCX                      | Antihistamines and adrenergics                      | 90   |
| Reversed-phase    | $\mu$ Bondapak C <sub>18</sub> | Catecholamines and phenylephrine                    | 96   |
|                   |                                | Phentolamine  | 97   |
|                   |                                | Dopamine and norendrenaline                         | 93   |
|                   |                                | Pseudoephedrine and chlorpheniramine                | 95   |
|                   |                                | Cough expectorants                                  | 94   |
|                   | Partisil ODS                   | Catechol derivatives                                | 92   |
|                   | Corasil C <sub>18</sub>        | Antihistamines and antitussives                     | 91   |
|                   | $\mu$ Bondapak: phenyl         | Pseudoephedrine, triprolidine, chlorpheniramine     | 100  |
|                   | Corasil phenyl                 | Antihistamines and antitussives                     | 91   |
| Miscellaneous     | $\mu$ Bondapak CN              | Catecholamines                                      | 84   |
|                   |                                | Phenylephrine, phenylpropanolamine, brompheniramine | 98   |
|                   | Amberlite XAD-2                | Cough/cold syrups                                   | 99   |

Table 2. HPLC techniques used for the analysis of catecholamines and related compounds.



Adsorption onto silica has been used by Jane (88) for a wide range of drugs including adrenergics like ephedrine. Cashman et al. (89) used silica and also a strong anion exchanger to separate amphetamines and a few other phenethylamines, whilst good separation of the components of cough/cold remedies has been achieved on strong cationic exchangers (90). Knox and Jurand (84) used three types of HPLC for catecholamines and their metabolites and concluded that "soap chromatography" was the technique of choice, giving the most efficient columns and greatest sensitivity of detection. Several other workers have used reversed phase HPLC for similar compounds although ion-pairing with long chain surfactants has not always been found necessary (91-98).

Biological samples are usually pre-treated to remove the endogenous substances which tend to accumulate on the columns and shorten their useful life. A similar problem might be expected in the analysis of pharmaceutical syrups. These also contain a great many adjuvants although their nature is somewhat different from those compounds encountered in biological samples. Mohammed and Cantwell (99) succeeded in directly analysing pharmaceutical syrups by using a pre-column of anionic exchange material before the main analytical column of nonionic resin. The precolumn retained unwanted substances, including colourings and preservatives, whilst the drugs of interest were washed onto the main column. The precolumn was then washed while the main column was being developed. Such a system requires a fairly complicated valve system and two solvent pumps and is outside the scope

of many analytical establishments.

Bachman has also analysed pharmaceutical syrups by HPLC but he extracted the drugs by shaking the diluted syrup with diatomaceous earth (100). This was separated and eluted with water-saturated chloroform. Aliquots of this solution were applied to a reversed-phase HPLC column for analysis. Direct analysis of cough expectorants without any pre-treatment has been achieved by injecting samples of diluted syrup onto a reversed-phase column and eluting with methanolic potassium dihydrogen phosphate solution (94).

In the development of a stability indicating assay for salbutamol, the TLC separation was taken as a starting point, since it is sometimes claimed that a TLC separation can be transferred to HPLC with very little modification to the mobile phase (101, 102). A 25 cm silica gel (Partisil 10) column was used with the TLC solvent (79) as the mobile phase. The detection wavelength was 254 nm and 10  $\mu$ l injections were made via a loop valve.

The traces obtained are shown in Fig. 14. The peaks are quite sharp but the baseline was not stable and replicate injections showed a marked loss of height. Calculation of the plate number (Equation 11) revealed that the column efficiency had fallen to 25% of its starting value after four injections. Some separation of the components of a degraded salbutamol sulphate solution was achieved (Fig. 15) but resolution was far from complete.

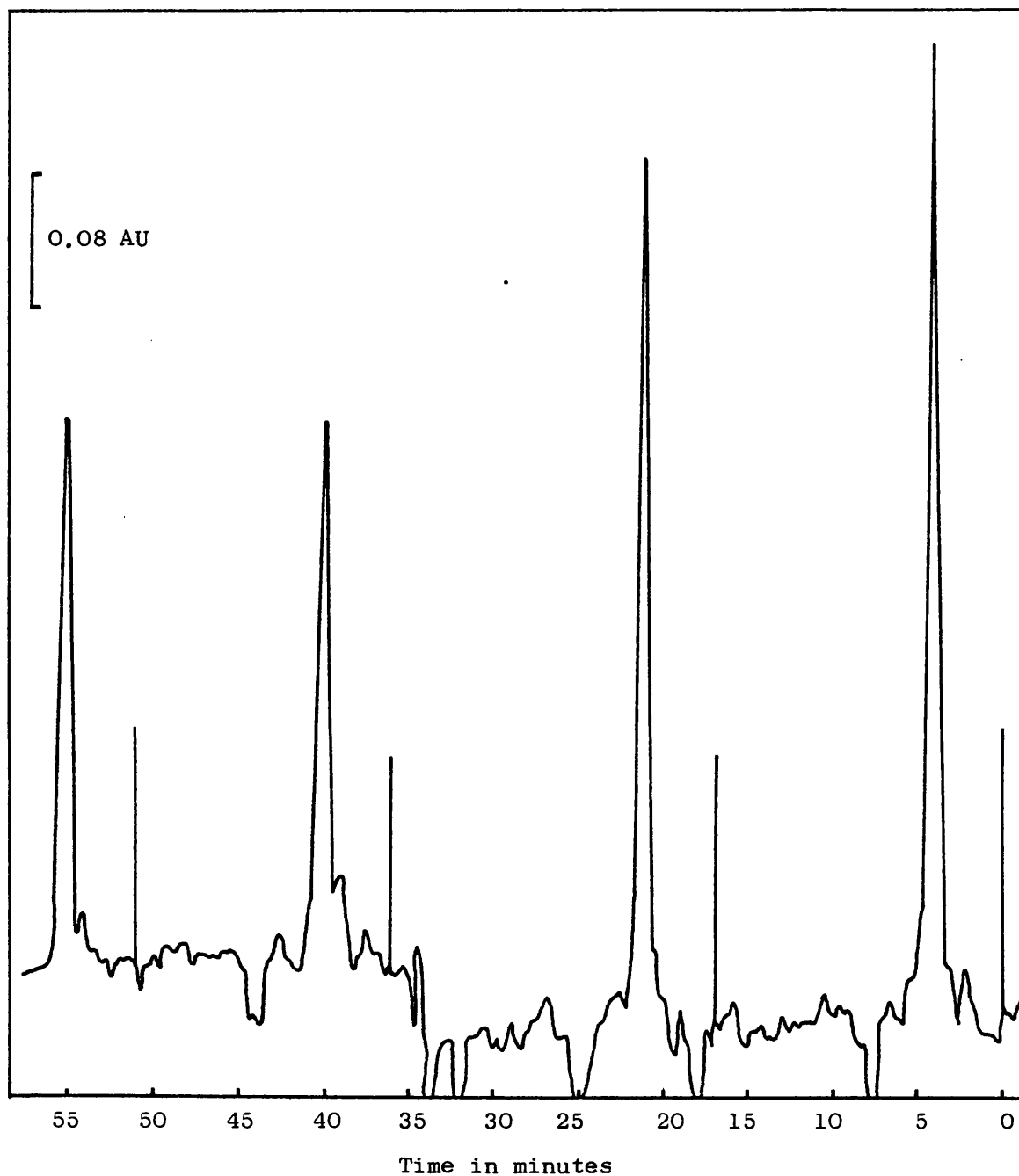


Fig. 14. HPLC traces of 0.2% salbutamol sulphate solution

Column: 25 cm Partisil 10

Solvent: Ethyl acetate: isopropanol:water:ammonia  
(50:30:16:4)

Flow rate: Approx.  $1.5 \text{ ml min}^{-1}$

Detection: UV 254 nm

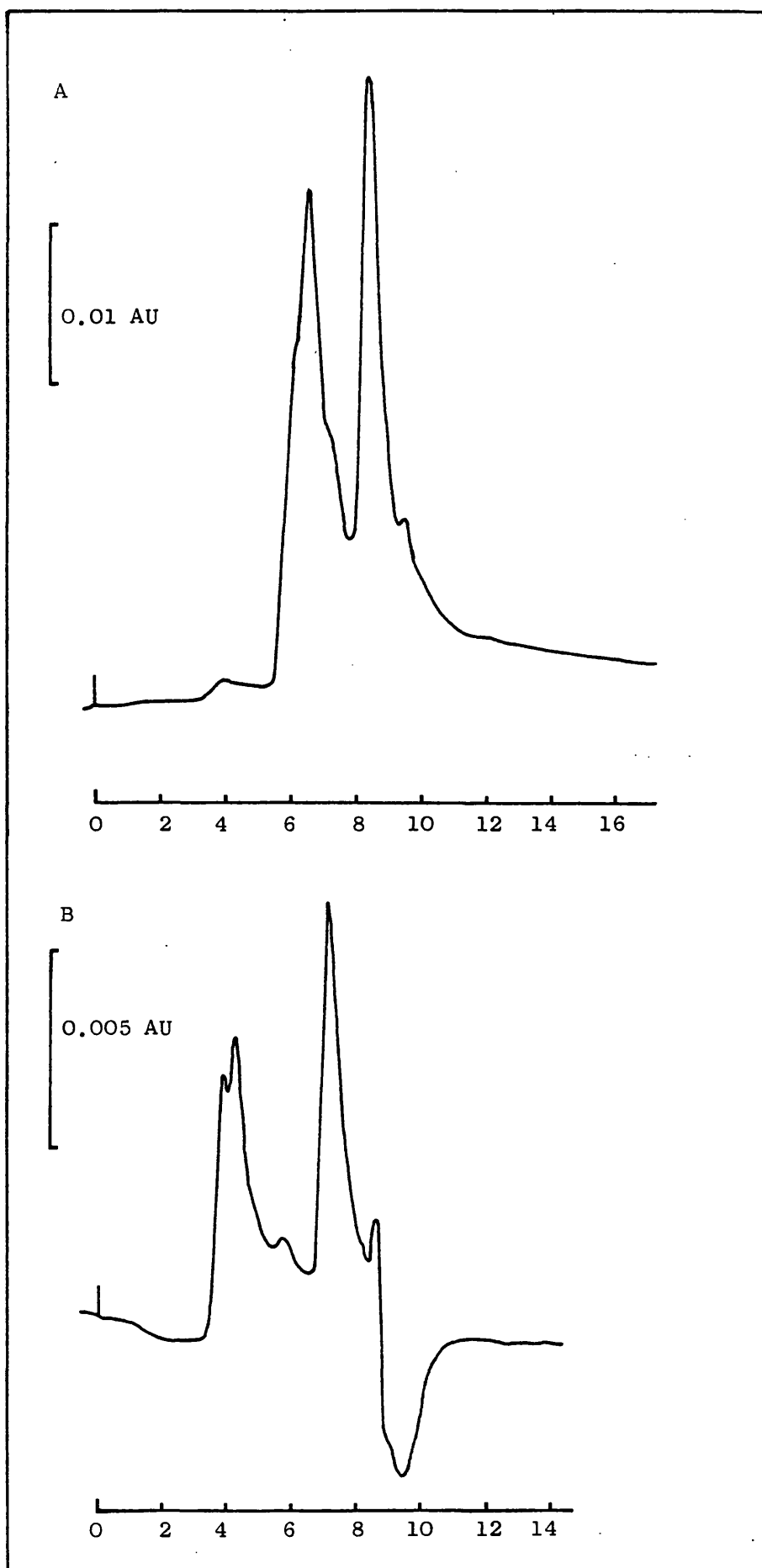


Fig. 15. HPLC traces of degraded 0.2% salbutamol sulphate solutions  
Time scale in minutes

A Conditions as Figure 14.

B Conditions as Figure 14 but ammonia content in solvent  
reduced to 1%.

The high content of ammonia in the TLC solvent (4%) was thought to be responsible for the steady loss of column efficiency. Silica is unstable at high pH and so strongly alkaline mobile phases are not recommended for HPLC (81, 92). Reduction of the ammonia content to 1% produced better resolution of degraded samples although this was still not satisfactory (Fig. 15). The large negative peak at approximately 9.5 minutes was due to the water present in the sample and could have masked additional degradation peaks.

A series of investigations were then carried out using mobile phases of different composition. The isopropanol was replaced by methanol in some cases, to increase the polarity of the solvent and the ratio of ethyl acetate to alcohol was varied. Acidic mobile phases were also tried in an attempt to prevent the progressive fall in plate number which was still

| Component      | Mobile phase |    |    |    |    |     |    |     |
|----------------|--------------|----|----|----|----|-----|----|-----|
|                | A            | B  | C  | D  | E  | F   | G  | H   |
| Ethyl acetate  | 100          | 80 | 70 | 80 | 80 | 40  | 60 | 100 |
| Methanol       | 68           | 88 | 88 | -  | -  | -   | -  | 100 |
| Isopropanol    | -            | -  | -  | 88 | 88 | 108 | 98 | -   |
| Water          | 32           | 32 | 40 | 32 | 32 | 52  | 42 | -   |
| 0.880 ammonia  | 2            | 2  | 2  | 2  | -  | -   | -  | 2   |
| Sulphuric acid | -            | -  | -  | -  | 1  | 1   | 1  | -   |

Table 3. The composition of HPLC mobile phases used during attempts to separate degraded salbutamol sulphate solutions on a 25cm Partisil 10 column.

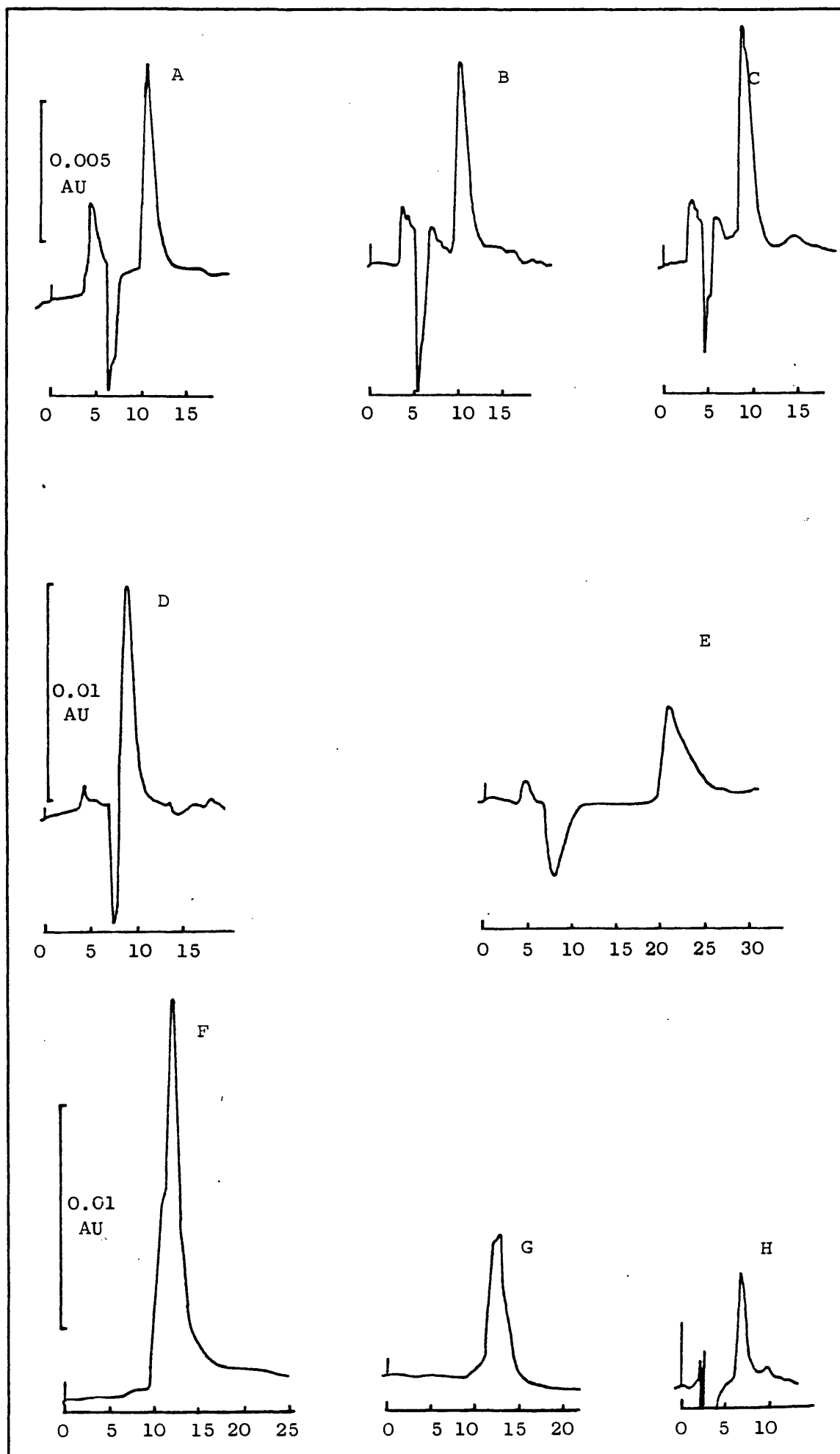


Fig. 16. HPLC traces of degraded 0.2% salbutamol sulphate solutions. Solvent compositions as Table 3. Other conditions as Fig. 14. Time scale in minutes.

being experienced. The compositions are summarised in Table 3 and the results in Fig. 16. None of the modifications significantly improved the separation of the degradation products. In most cases the large negative peak due to the water in the sample appeared in the middle of the chromatogram and could easily have obscured peaks due to breakdown products. The acidic solvents proved to be totally unsuitable for salbutamol as can be seen from Fig. 16.

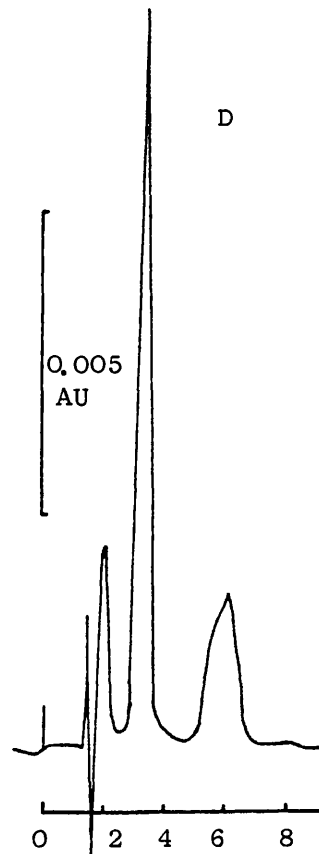
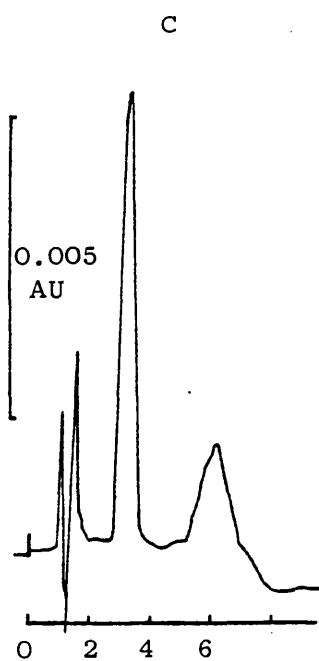
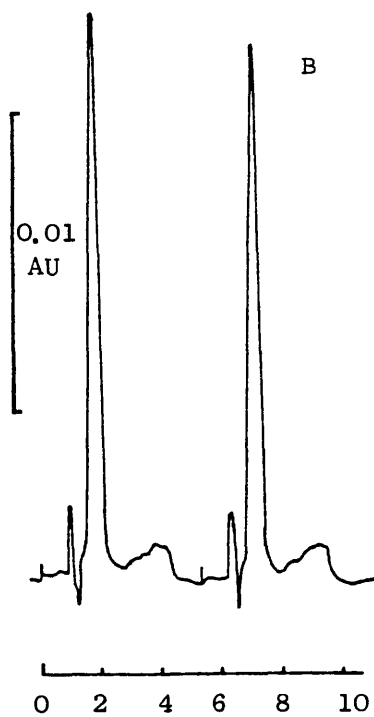
It seemed apparent that a satisfactory separation would not be achieved on silica columns and so Partisil-10 SCX, a strong cationic exchanger, was examined. The mobile phase consisted of 10% methanol in citrate-phosphate buffer pH 7.4, a system which had been used successfully for stability studies on phenylephrine in our laboratories (104). The peaks which were obtained were very small and poorly resolved and it seemed unlikely that improvements could easily be made. Consequently, recourse was made to reversed-phase ion-pair or "soap chromatography".

The first column tested was 20 cm in length but this proved too great because it resulted in very long retention times and short, broad peaks. The pairing ion used was dioctyl sodium sulphasuccinate (Mannoxol OT) at a concentration of 0.01%. Typical traces, obtained using a high percentage of methanol to reduce the retention times, are shown in Fig. 17. A smaller pairing ion would be expected to bind less strongly to the column and so have a shorter retention time. Perchloric acid (0.01M), which has been used as a pairing ion

Fig. 17. HPLC traces of degraded 0.2% salbutamol sulphate solutions

- A. Column: 20 cm Spherisorb S5 ODS  
Solvent: 75% methanol, 0.01% Mannoxol OT,  
0.04% sulphuric acid  
Flow rate: Approx.  $1.5 \text{ ml min}^{-1}$   
Detection: uv 254 nm
- B Column: 10 cm Spherisorb S5 ODS  
Solvent: 33% methanol, 0.01M perchloric acid  
Flow rate: Approx.  $1.5 \text{ ml min}^{-1}$   
Detection: uv 254 nm
- C. As B except Solvent: 37.5% methanol, 0.004%  
sodium dodecyl sulphate,  
0.02% sulphuric acid
- D. As B except Solvent: 37.5% methanol, 0.004%  
sodium dodecyl sulphate,  
0.06% perchloric acid.

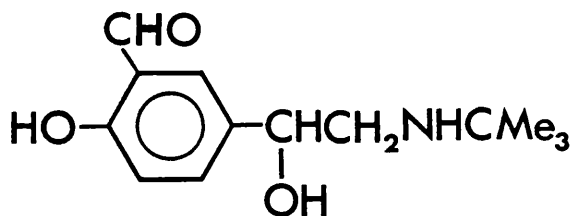




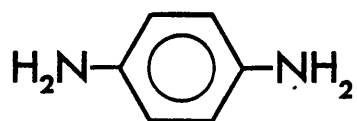
by some workers (81) was therefore studied, as a 50% methanolic solution. However, no peaks eluted in less than 20 minutes and a similar problem was encountered with sodium dodecyl sulphate (0.004%). A small percentage of sulphuric acid was used with the latter to ensure that the salbutamol was fully ionised and therefore able to form an ion-pair. A 10 cm column was prepared in order to reduce the retention times.

The 10 cm Spherisorb S5 ODS column was tested using perchloric acid or sodium dodecyl sulphate as the pairing ion and the results are shown in Fig. 17. Resolution was better using the surfactant as the pairing ion but since Gloor and Johnson (81) have reported that a mixture of pairing ions can produce improved separations, a combination of perchloric acid and sodium dodecyl sulphate was tested. The result was a much sharper separation with improved peak heights as demonstrated in Fig. 17. Sensitivity could be increased by changing the detection wavelength to 278 nm ( $\lambda_{\text{max}}$  for salbutamol) but the general nature of the chromatogram did not change, indicating that degradation products with a markedly different absorption spectrum were not being missed.

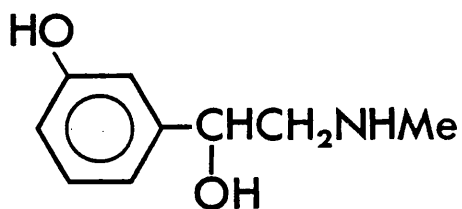
Having established an adequate separation, an internal standard was required to compensate for peak variations due to irregularities in the solvent flow rate or in the injection volume. Several compounds (Fig. 18) were tested and they are listed, together with their capacity ratios in Table 4. Ephedrine proved to be the most promising, being well separated from salbutamol but still giving a good, sharp



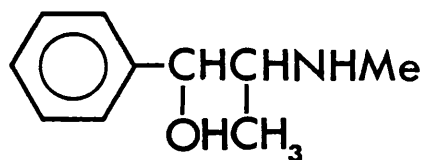
AH 6368



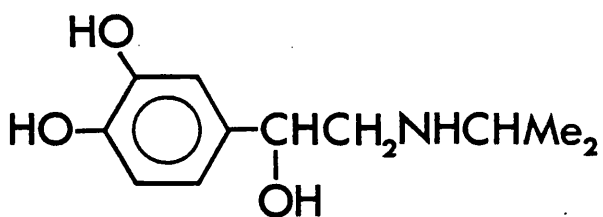
Phenylene diamine



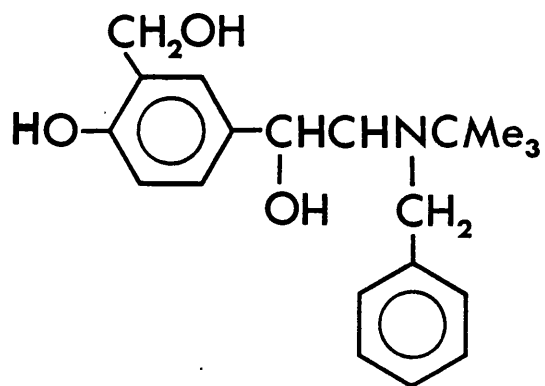
Phenylephrine



Ephedrine



Isoprenaline



"Benzyl salbutamol"

Fig. 18. Compounds tested as potential internal standards  
for the HPLC assay of salbutamol sulphate.

| Compound                 | Retention time  | k'<br>Capacity ratio | Comments                |
|--------------------------|-----------------|----------------------|-------------------------|
| Salbutamol               | 3 mins          | 1.79                 |                         |
| AH 6368                  | 6 mins 7 secs   | 4.22                 |                         |
| Phenylephrine            | 2 mins 37 secs  | 1.22                 |                         |
| Isoprenaline             | 2 mins 32 secs  | 1.20                 |                         |
| Phenylene<br>diamine     | 9 mins 45 secs  | 5.74                 | Unstable in<br>solution |
| Ephedrine                | 4 mins 35 secs  | 3.19                 |                         |
| 'Benzyl salbut-<br>amol' | 10 mins 30 secs | 7.46                 | Very broad peak         |

Table 4. HPLC data of potential internal standards.

peak. When added to mildly degraded solutions, it eluted between salbutamol and the next degradation peak as indicated in Fig. 19. However, in more extensively degraded solutions, an additional peak develops in this position and this would therefore interfere with the internal standard (Fig. 19). Compounds which would elute after all the breakdown products had such long retention times that the peak was too broad to be analytically useful. It was necessary, therefore, either to manage without an internal standard or to separate the components of the mixture more efficiently.

The latter option is to be preferred and this led to the investigation of SP-Sephadex - C25. This material is a cationic exchanger and has been used by the analysts in the industrial laboratories to extract salbutamol from syrup formulations prior to assay. In the method used, diluted syrup is washed,

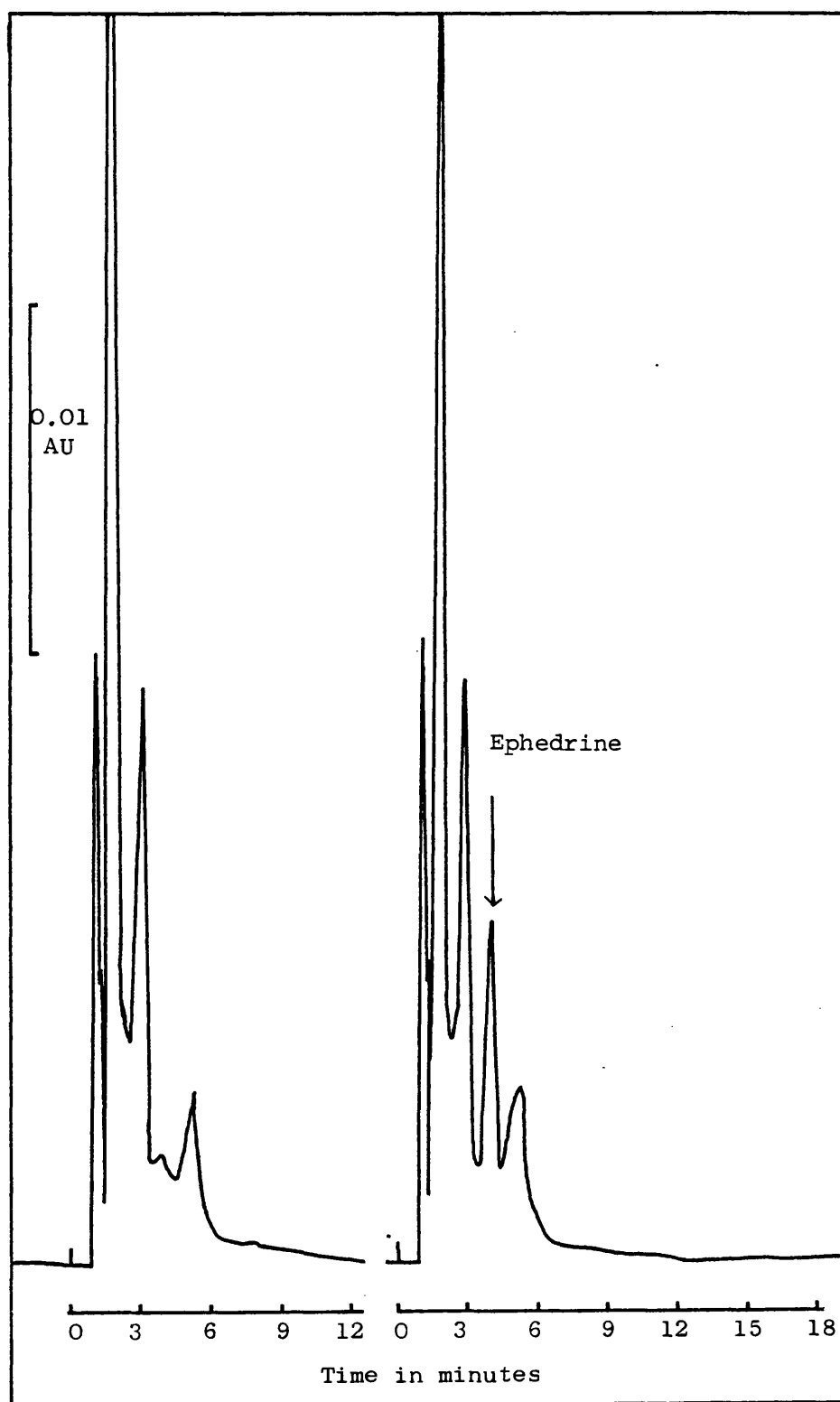


Fig. 19. HPLC traces of degraded 0.1% salbutamol sulphate solutions, showing interference of new degradation peak with ephedrine, the internal standard. Conditions as Fig. 17D.

with water, through a column of SP-Sephadex gel. This removes the sugars and other non-basic compounds whilst the drug, which is basic and ionised, is retained by the ion-exchange column. It is then eluted using an alkaline solvent, the eluant being collected for assay.

An initial experiment was conducted in which 5 ml of a degraded solution of 0.1% salbutamol sulphate (estimated to contain approximately 50% residual drug) was applied to a short SP-Sephadex column. The column was washed with water, the eluant collected and its uv absorption spectrum determined at intervals. Washing was continued until the eluant showed no absorption in the range 200 - 400 nm. The eluting solvent was then changed to 1% 0.880 ammonia solution and the procedure repeated. Samples from both the non-basic and basic fractions showing uv absorption between 200 nm and 400nm were injected onto the HPLC column and the results obtained are depicted in Fig. 20.

Fig. 20 clearly shows that the basic fraction contains far fewer breakdown products than the original solution, and is consequently much better resolved. The small peak which caused the problem with the internal standard elutes in the non-basic fraction and so is readily removed from the samples. However, the elution from the ion-exchange column necessarily dilutes the sample, requiring a higher sensitivity setting on the HPLC detector. The sensitivity could also be increased by using a wavelength of 278 nm instead of 254 nm but ephedrine does not absorb significantly at this wavelength

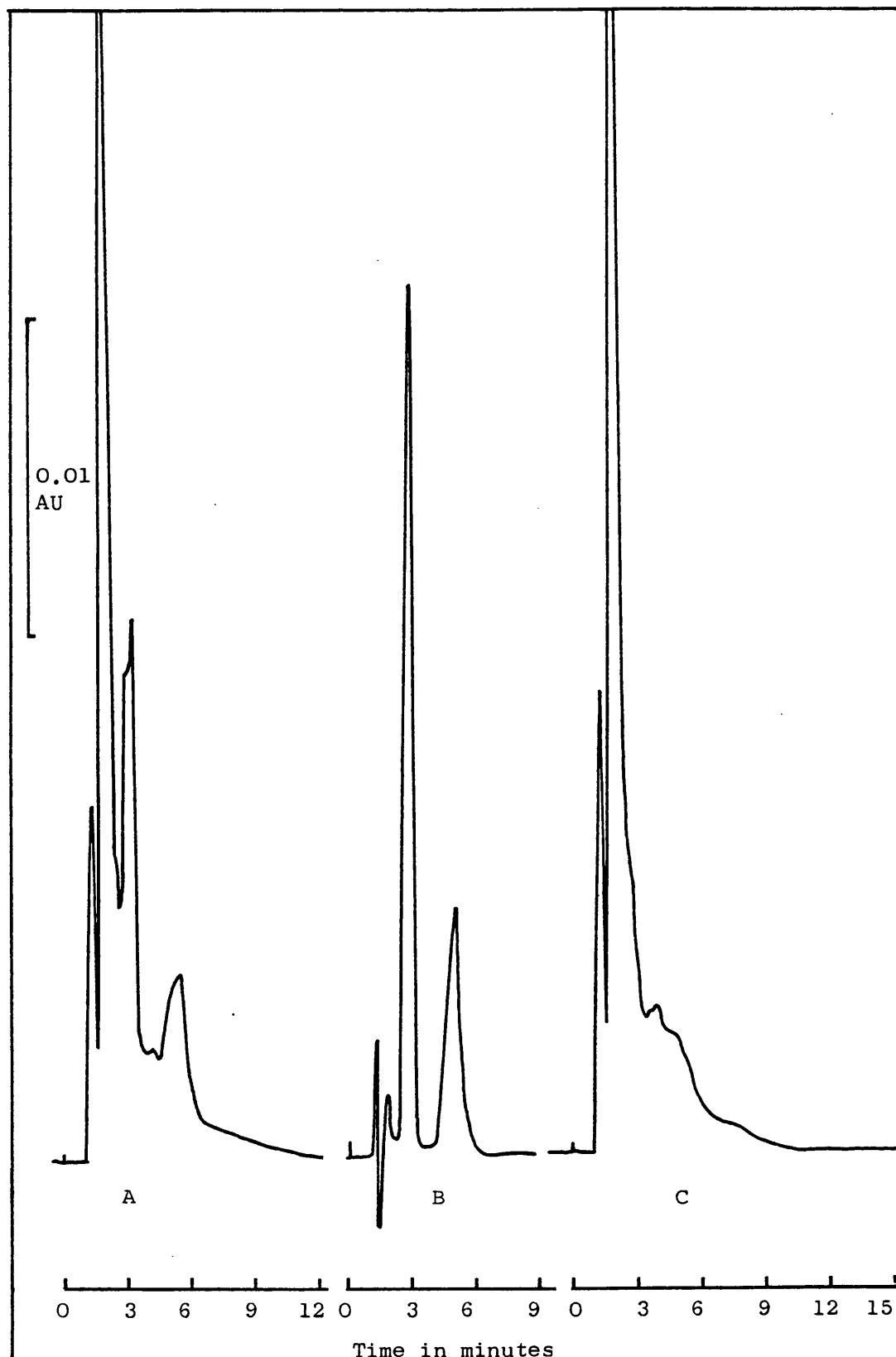


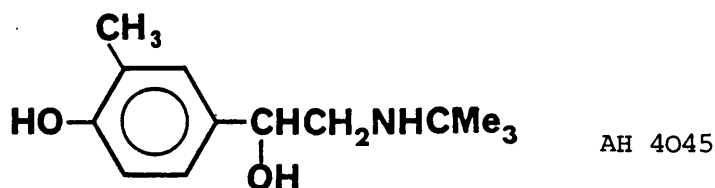
Fig. 20.A.HPLC trace of degraded 0.1% salbutamol sulphate solution.

B.HPLC trace of basic fraction of same solution eluted from SP-Sephadex column

C.HPLC trace of non-basic fraction of same solution eluted from SP-Sephadex column.

Conditions as Fig. 17D.

and would therefore be unsuitable as internal standard. However, AH 4045 has an absorption spectrum similar to that of salbutamol and absorbs strongly at 278 nm. When tested, it



gave a good, sharp peak at the desired position in the chromatogram and so was adopted as internal standard.

Baseline resolution between salbutamol and AH 4045 was just possible using the methanolic solvent system but better resolution was found when acetonitrile was used as the organic modifier. Typical traces are shown in Fig. 21. An improvement in the column packing facilities in the laboratory enabled a column with a higher plate number to be used for the subsequent analytical work.

The HPLC system was therefore finally established as:-

Column: 10 cm x 0.4 cm Spherisorb S5 ODS

Mobile phase: 24% acetonitrile

0.004% sodium dodecyl sulphate

0.06% perchloric acid

Flow rate: Approximately 1.5 ml min<sup>-1</sup>

Detection: uv absorption at 278 nm

Internal  
standard: 0.02% AH 4045



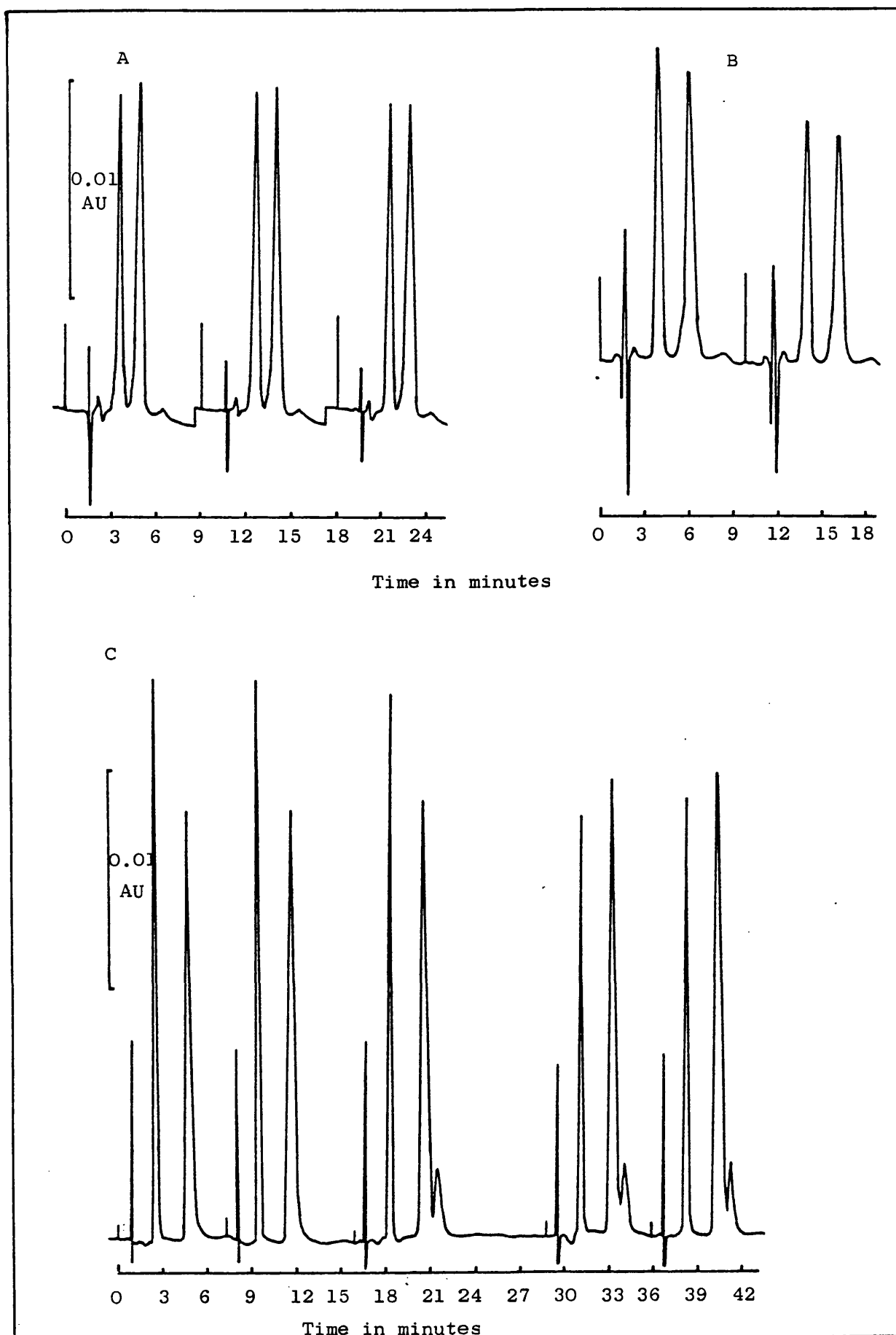


Fig. 21. A. HPLC trace of 0.5% salbutamol sulphate solution. Conditions as Fig. 17D.  
 B. HPLC trace of 0.5% salbutamol sulphate solution. Methanol replaced by acetonitrile but other conditions as Fig. 17D.  
 C. HPLC traces of progressively degraded salbutamol sulphate solutions. Conditions as p. 66.

## 2.2 Verification of the HPLC Assay

The HPLC assay procedure efficiently separated salbutamol ( $k'=1.8$ ) and the internal standard, AH 4045 ( $k'=4.1$ ) in spite of the fact that the structural difference between them is very small. It therefore seemed unlikely that any degradation products would fail to be separated from salbutamol. However, in order to verify this, the components of an approximately 50% degraded solution of salbutamol sulphate were separated by preparative TLC, as described in Section 2.7. The bands were visualised under uv light, the silica in each was carefully scraped off the plate and the adsorbed compounds were eluted with methanol. Aliquots of the resultant solution, suitably diluted if necessary, were injected onto the HPLC column.

All the major degradation products were tested in this way and all either eluted well away from the salbutamol peak in the chromatogram (Fig. 22) or failed to produce a significant peak. It was not possible to correlate the HPLC retention time with the  $R_f$  value on TLC.

The assay procedure therefore is believed to be stability indicating.

## 2.3 Sample Preparation and HPLC Assay

Short columns of SP-Sephadex C-25 were prepared by soaking the dry ion-exchange material in 1.0M ammonium chloride solution overnight. The supernatant liquid was decanted off

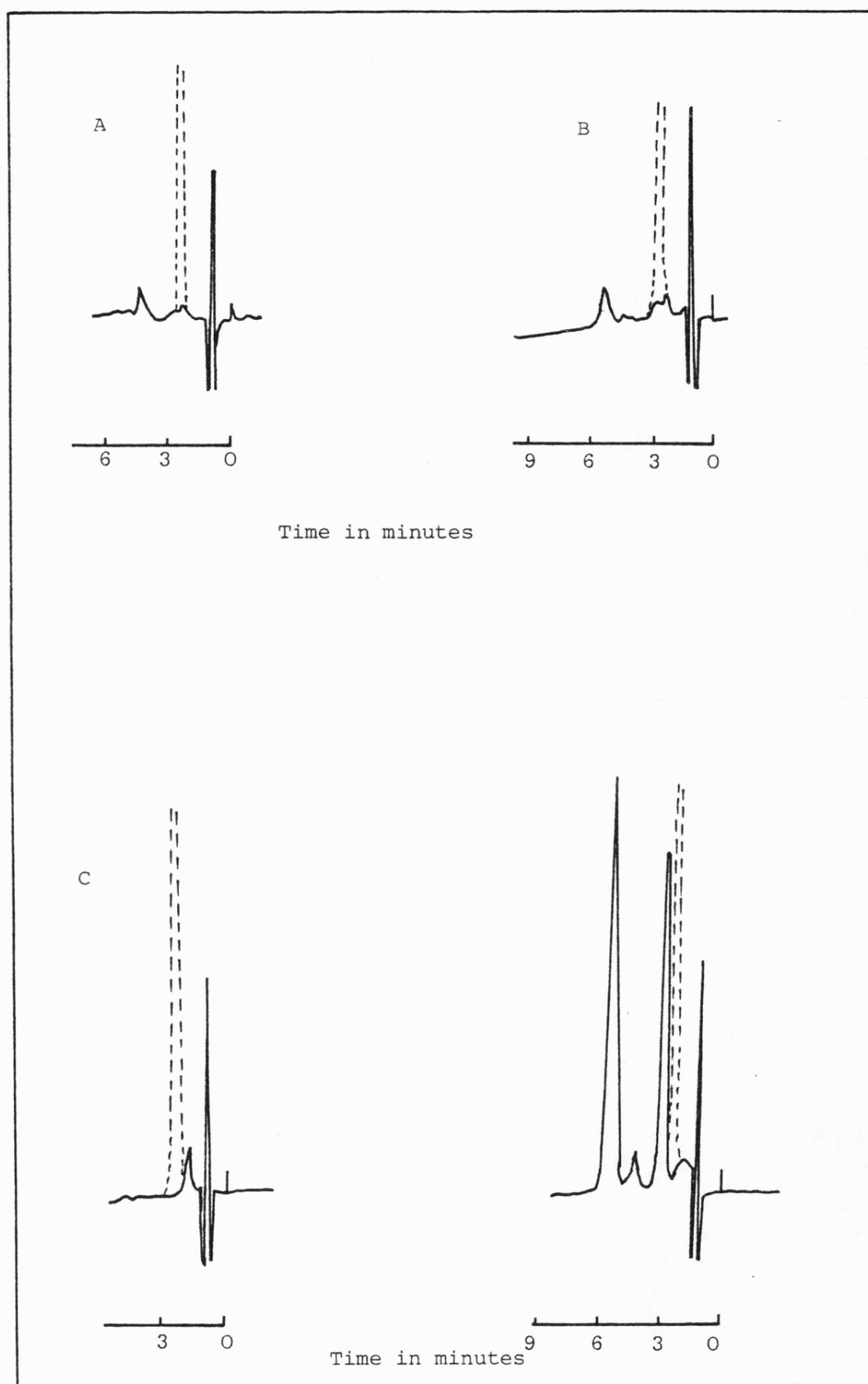


Fig. 22. HPLC traces of degradation products eluted with methanol from preparative TLC plates. Conditions as Fig. 21C.

A. Methanol

B. Degradation product Rf 0.7

C. Degradation product Rf 0.2.

D. Degradation product Rf 0.75-0.8

Dotted lines indicate position of salbutamol.

and the swollen gel resuspended in distilled water. This was repeated three more times before pouring the slurry into the glass columns. The Sephadex gel was supported by a small plug of glass wool and sufficient slurry was poured into each column to give a depth of approximately 6 cm gel. The empty column above the Sephadex will subsequently be referred to as the column volume. The Sephadex column was washed *in situ* with two column volumes of distilled water before use.

Samples from kinetic experiments were diluted, if necessary, to give a nominal concentration of 0.1% salbutamol sulphate. 2 ml of this solution (equivalent to 2 mg salbutamol sulphate) were applied to the prepared Sephadex column and allowed to run into the gel. The column was then washed with at least two column volumes of distilled water to remove non-basic compounds. In the case of sugar solutions, three column volumes were used to ensure complete removal of residual sugar. The retained, basic compounds were then eluted with dilute ammonia solution which was prepared by diluting 0.880 ammonia solution 1 in 100 with distilled water.

A 0.2% stock solution of the internal standard was prepared by dissolving 100 mg AH 4045 in 1% sulphuric acid to 50 ml, and this solution was then stored in a refrigerator for no longer than one month. The eluant from each Sephadex column was collected up to the graduation in a 10 ml volumetric flask containing 1 ml internal standard solution. 10  $\mu$ l aliquots of this solution were injected onto the HPLC

column for assay. The sample preparation procedure is summarised in Fig. 23.

The mobile phase for the HPLC assay consisted of approximately 24% acetonitrile containing 0.06% perchloric acid and 0.004% sodium dodecyl sulphate. This was prepared by mixing 240 ml acetonitrile with 760 ml water and degassing the mixture under vacuum on a rotary evaporator. To this were added 4 ml aqueous 1% sodium dodecyl sulphate solution and 10 ml aqueous 6% perchloric acid solution.

A flow rate of approximately  $1.5 \text{ ml min}^{-1}$  was used and a detection wavelength of 278 nm. Typical traces are shown in Fig. 21. Peak areas were measured by the Minigrator computing integrator and the ratio of the salbutamol peak area to that of the internal standard was calculated for each injection. At least three replicate injections of each sample were made.

The system was calibrated by using a series of solutions of salbutamol sulphate, having concentrations in the range 0.02% to 0.15%. Each solution was subjected to the same Sephadex pre-treatment as the stability samples and a typical set of results is given in Table 5. Three such calibrations were performed on different days and the mean data submitted to least squares regression analysis (Appendix 1); the results are summarised in Table 6.

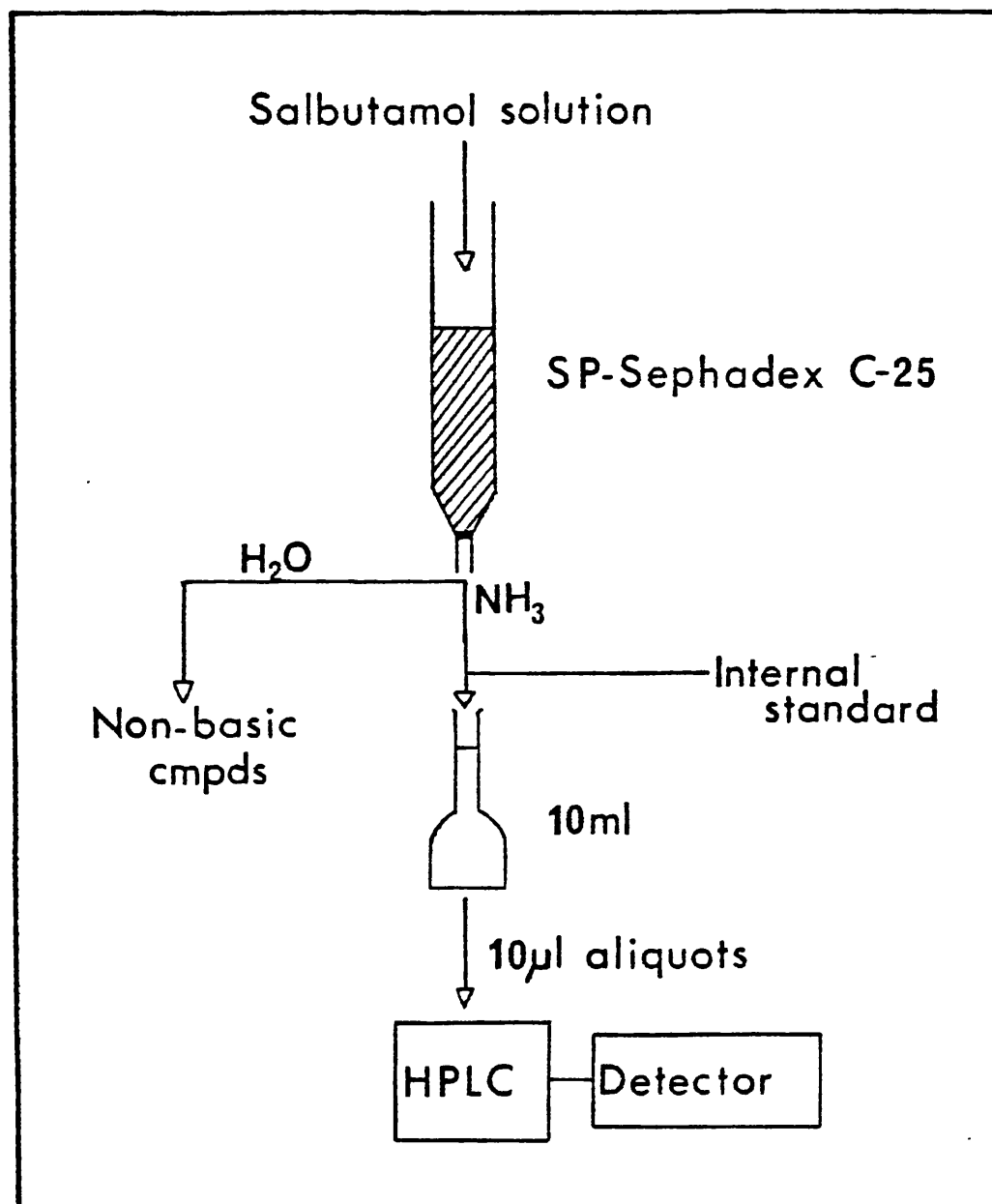


Fig. 23. Schematic representation of sample preparation procedure for stability samples prior to HPLC assay

| Concentration of<br>salbutamol sulphate<br>% w/v | Mean peak<br>area ratio | Number of<br>injections | Standard<br>deviation | Coefficient<br>of variation |
|--|-------------------------|-------------------------|-----------------------|-----------------------------|
| 0.02   | 0.221                   | 6                       | 0.003                 | 1.36%                       |
| 0.05   | 0.518                   | 8                       | 0.004                 | 0.79%                       |
| 0.08   | 0.811                   | 7                       | 0.009                 | 1.11%                       |
| 0.10   | 1.006                   | 8                       | 0.004                 | 0.41%                       |
| 0.15   | 1.546                   | 8                       | 0.007                 | 0.47%                       |

Table 5. Typical calibration data for the HPLC assay of  
salbutamol sulphate

| Calibration<br>number | Slope  | Intercept on<br>y axis | Std.Dev.<br>of slope | Std.Dev.of<br>intercept | Correlation<br>coefficient |
|-----------------------|--------|------------------------|----------------------|-------------------------|----------------------------|
| 1                     | 10.153 | 0.0080                 | 0.156                | 0.014                   | 0.9996                     |
| 2                     | 9.105  | -0.0059                | 0.159                | 0.011                   | 0.9996                     |
| 3                     | 9.031  | 0.0028                 | 0.204                | 0.013                   | 0.9992                     |

Table 6. Details of calibration lines for the HPLC assay of  
salbutamol sulphate

The slopes of the calibration lines in Table 6 vary by about 10% and are therefore significantly different. Because of this daily variation in column performance, three standard solutions of salbutamol sulphate were always assayed together with the stability samples. These standards (0.1%, 0.05%, 0.02%) were treated in exactly the same way as the other samples and spanned the range of drug concentration in those samples. They were interspersed randomly through the day's samples for assay and a calibration line was calculated from their results using least squares regression analysis (Appendix 1). The intercept of this calibration line was never of significance and so the concentration of salbutamol sulphate in the kinetic samples could be calculated simply from the value of the slope. A typical day's results are given in Table 7, together with the method of calculation.

The daily variation in the calibration lines showed random scatter as demonstrated by the data in Appendix 2. However, the performance of the HPLC column remained stable during the course of a working day since replicate injections gave the same result when injected at the beginning and end of the day (Table 8).

| Concentration of<br>salbutamol sulphate | Beg. of day's assays |                        | End of day's assays |                        |
|---|----------------------|------------------------|---------------------|------------------------|
|   | Peak area<br>ratio   | Coeff. of<br>variation | Peak area<br>ratio  | Coeff. of<br>variation |
| 0.10%                                   | 1.019                | 0.57%                  | 1.017               | 0.54%                  |
| 0.02%                                   | 0.218                | 0.38%                  | 0.218               | 1.85%                  |

Table 8. Reproducibility of HPLC response over a working day.



| Sample      | Peak Area Ratios                    | Mean   | Standard Deviation | Coefficient of variation | % Salbutamol sulphate remaining |
|-------------|-------------------------------------|--------|--------------------|--------------------------|---------------------------------|
| 0.10% std.  | 0.920,0.916,0.915,0.924,0.909       | 0.9168 | 0.006              | 0.61%                    | -                               |
| 85° Initial | 0.934,0.930,0.924,0.935             | 0.931  | 0.005              | 0.54%                    | 102.69                          |
| 85° 4 hrs   | 0.873,0.859,0.870,0.867,0.865,0.859 | 0.8655 | 0.006              | 0.66%                    | 95.47                           |
| 0.05% std   | 0.455,0.448,0.454,0.453             | 0.4525 | 0.003              | 0.69%                    | -                               |
| 85° 9 hrs   | 0.784,0.789,0.786                   | 0.786  | 0.003              | 0.32%                    | 86.70                           |
| 85° 22 hrs  | 0.606,0.614,0.613,0.609             | 0.6105 | 0.004              | 0.61%                    | 67.34                           |
| 0.02% std   | 0.193,0.194,0.197,0.190             | 0.1935 | 0.003              | 1.49%                    | -                               |

|   |                    |
|---|--------------------|
| Calibration line: Intercept = 0.0072  | Std. Dev. = 0.0113 |
| Slope = 9.066   | Std. Dev. = 0.173  |
| Correlation coefficient, r = 0.9998   |                    |
| Percentage salbutamol sulphate remaining = $\frac{\text{Mean}}{\text{Slope}} \times 1000$ |                    |

Table 7. Typical daily HPLC calibration and assay results for kinetic studies on the degradation of salbutamol sulphate solutions.

The one anomaly which was noted was in the result obtained from the first injection of a new solution. This was nearly always markedly different from subsequent results and so the first injection was ignored for assay purposes. Examples from the assay results for a 0.5% salbutamol sulphate solution at pH 7 and 70°, are given in Table 9.

| Sample   | Peak Area Ratios                           | Mean  | Std.Dev. | Coeff. of variation |
|----------|--|-------|----------|---------------------|
| Initial  | (0.986), 0.994, 1.011, 0.996, 1.006, 0.991 | 0.999 | 0.009    | 0.85%               |
| 21 hours | (0.932), 0.892, 0.896, 0.885, 0.899, 0.898 | 0.894 | 0.006    | 0.64%               |
| 45 hours | (0.821), 0.856, 0.853, 0.851               | 0.851 | 0.006    | 0.65%               |
| 117 hrs  | (0.677), 0.654, 0.652, 0.652, 0.651        | 0.652 | 0.0013   | 0.19%               |

Table 9. Assay results showing anomalous result for first injection onto HPLC column. Mean values are calculated without the first result.

#### 2.4 Indoaniline Colorimetric Assay

The B.P.C. assay for salbutamol inhalers (73) was modified for use on the samples from kinetic experiments. The samples were diluted with water in order to obtain a nominal concentration of 0.1 mg per ml. Into a 50 ml separating funnel, 3 ml distilled water and 1 ml of diluted sample were pipetted. To this solution the following aqueous reagents were added, in order, mixing thoroughly after each addition:

4 ml 5% sodium bicarbonate solution

4 ml 0.1% 1-dimethylamino-4-aminobenzene sulphate solution

4 ml 8% potassium ferricyanide solution

All reagents were freshly prepared

The resultant solution was left to stand for 15 minutes away from direct sunlight and then extracted with 2 x 10 ml portions of chloroform which were collected in a 25 ml volumetric flask and made up to volume. The absorbance of the blue chloroform solution was read at 605 nm, according to the B.P.C. method.

A series of standard salbutamol sulphate solutions over the range 0.10% to 0.02% was used to construct Beer-Lambert plots to check the linearity of the response. Each solution was diluted 1 in 10 before use and the results are summarised in Table 10.

| Calibration<br>No. | Slope | Intercept | Std.Dev.<br>of slope | Std.Dev.<br>of<br>intercept | Corr .<br>coefficient |
|--------------------|-------|-----------|----------------------|-----------------------------|-----------------------|
| 1                  | 3.008 | 0.035     | 0.071                | 0.0047                      | 0.9992                |
| 2                  | 3.138 | 0.015     | 0.065                | 0.0043                      | 0.9994                |
| 3                  | 3.046 | 0.025     | 0.080                | 0.0052                      | 0.9990                |

Table 10. Details of the calibration lines for the colorimetric assay of salbutamol sulphate

Both the slopes and intercepts of the Beer-Lambert calibrations were subjected to statistical analysis using the Bartlett test (Appendix 1). The slopes gave a  $\chi^2$  value

of 1.722 and the intercepts a value of 8.873. The tabulated value at the 5% probability level is 5.991 and so the intercepts differ significantly but the slopes do not. This implies that the three calibrations form a family of parallel lines (Fig. 24).

It was noted, when using the assay, that the glassware became coated with a dark deposit, removable with "chromic acid". It is believed that this deposit on the walls of the cuvette increased the absorbance reading and produced the positive intercept in the Beer-Lambert plots. Small variations in the degree of this adsorption would account for the differences in the intercepts. In order to eliminate this difference when calculating stability results, the value of the intercept was calculated from the absorbance of a standard solution assayed with the stability samples. For this, the value of the slope was taken as 3.064, the mean of the three calibrations.

Since the calibration plots are linear, they have the general equation:

$$A = mc + b$$

where A = absorbance, m = slope, c = drug concentration, b = constant. For the standard solution, A, c and m are known and hence b, the intercept, can be calculated.

The approach was checked by calculating the intercept from each point on one of the calibration plots, using the mean slope value of 3.064. The values obtained are given in

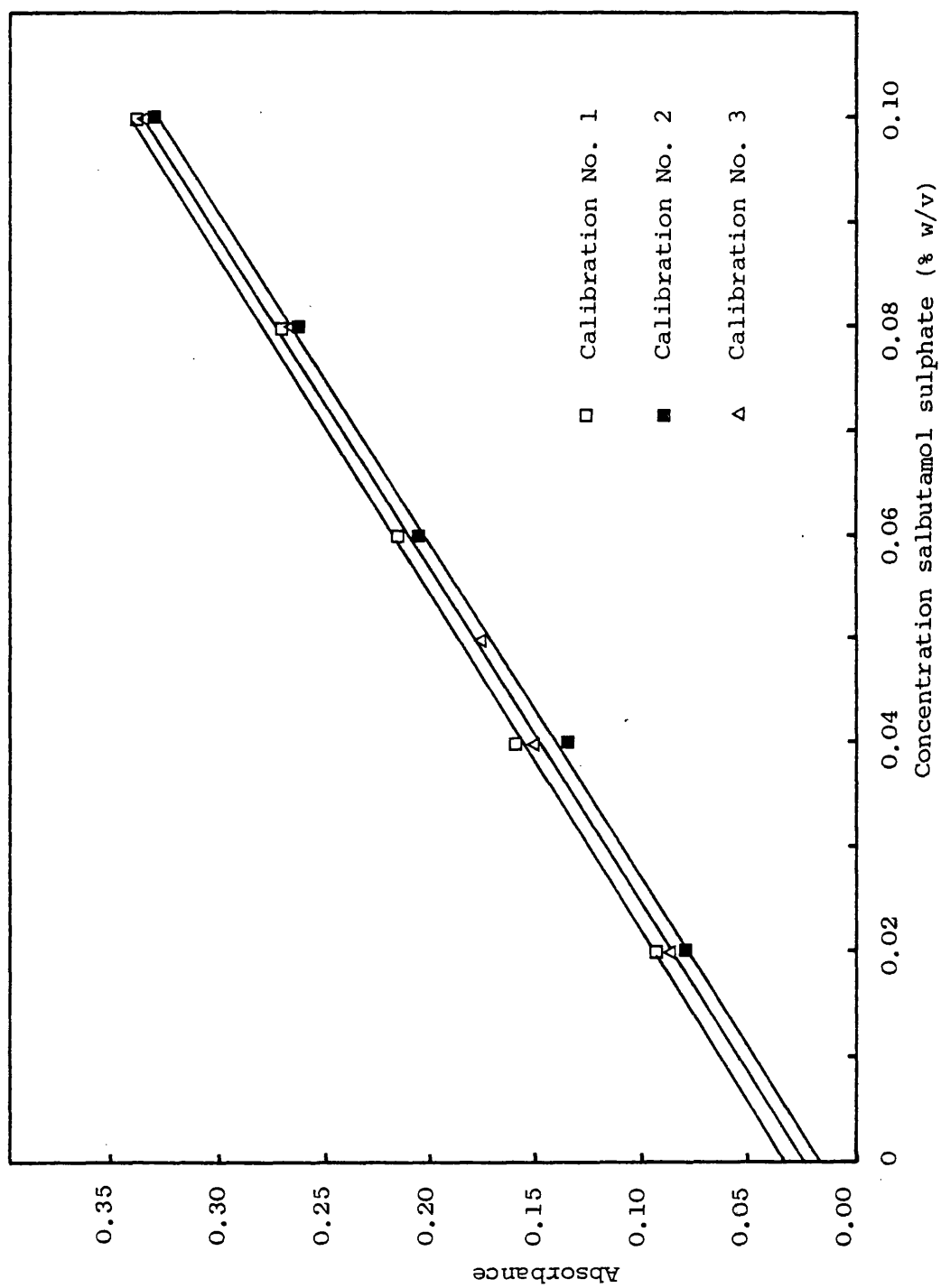


Fig. 24. Beer-Lambert calibration lines for the colorimetric assay of salbutamol sulphate

Table 11 and have a coefficient of variation of 13.6%. The mean value of 0.0314 is within about 10% of the regression line intercept of 0.035.

| Salbutamol sulphate<br>concentration (% w/v) | Calculated intercept |
|--|----------------------|
| 0.02   | 0.031                |
| 0.04   | 0.037                |
| 0.06   | 0.032                |
| 0.08   | 0.025                |
| 0.10   | 0.032                |

Table 11. Values of intercepts calculated for each point on calibration line No. 1 using value of 3.064 for the slope.

## 2.5 Comparison of Colorimetric and HPLC Assays

Samples from several stability experiments were assayed by both the indoaniline colorimetric method and the HPLC technique (Figs. 25-27, Tables 12-15). In all cases the indoaniline method gave appreciably higher results, indicating that it estimates some salbutamol degradation products as well as the intact drug. This was confirmed by spraying a developed TLC of degraded salbutamol solution (approximately 50% residual drug) with the indoaniline reagents. The pattern of spots visualised was the same as that revealed by the usual MBTH reagent (Section 2.6), the only difference being that the spots were blue instead of red.

| Sample<br>Time (hrs) | Percentage residual salbutamol sulphate |                   |
|----------------------|---|-------------------|
|                      | HPLC Assay                              | Indoaniline Assay |
| 0                    | 104.35                                  | 103.59            |
| 24                   | 87.67                                   | 98.69             |
| 48                   | 74.94                                   | 91.68             |
| 72                   | 65.93                                   | 85.80             |
| 143.3                | 49.28                                   | 73.56             |

Table 12. Comparison of assay techniques for 0.5% salbutamol sulphate solution, pH 7, 70<sup>o</sup> containing 0.05% EDTA

| Sample<br>Time (hrs) | Percentage residual salbutamol sulphate |                   |
|----------------------|---|-------------------|
|                      | HPLC Assay                              | Indoaniline Assay |
| 0                    | 96.52                                   | 99.08             |
| 24                   | 86.89                                   | N.D.              |
| 48                   | 79.91                                   | 98.05             |
| 72                   | 69.77                                   | 97.06             |
| 120                  | 57.75                                   | 89.88             |
| 168                  | 48.58                                   | 80.09             |

Table 13. Comparison of assay methods for 0.5% salbutamol sulphate solution, pH 7, 70<sup>o</sup>.

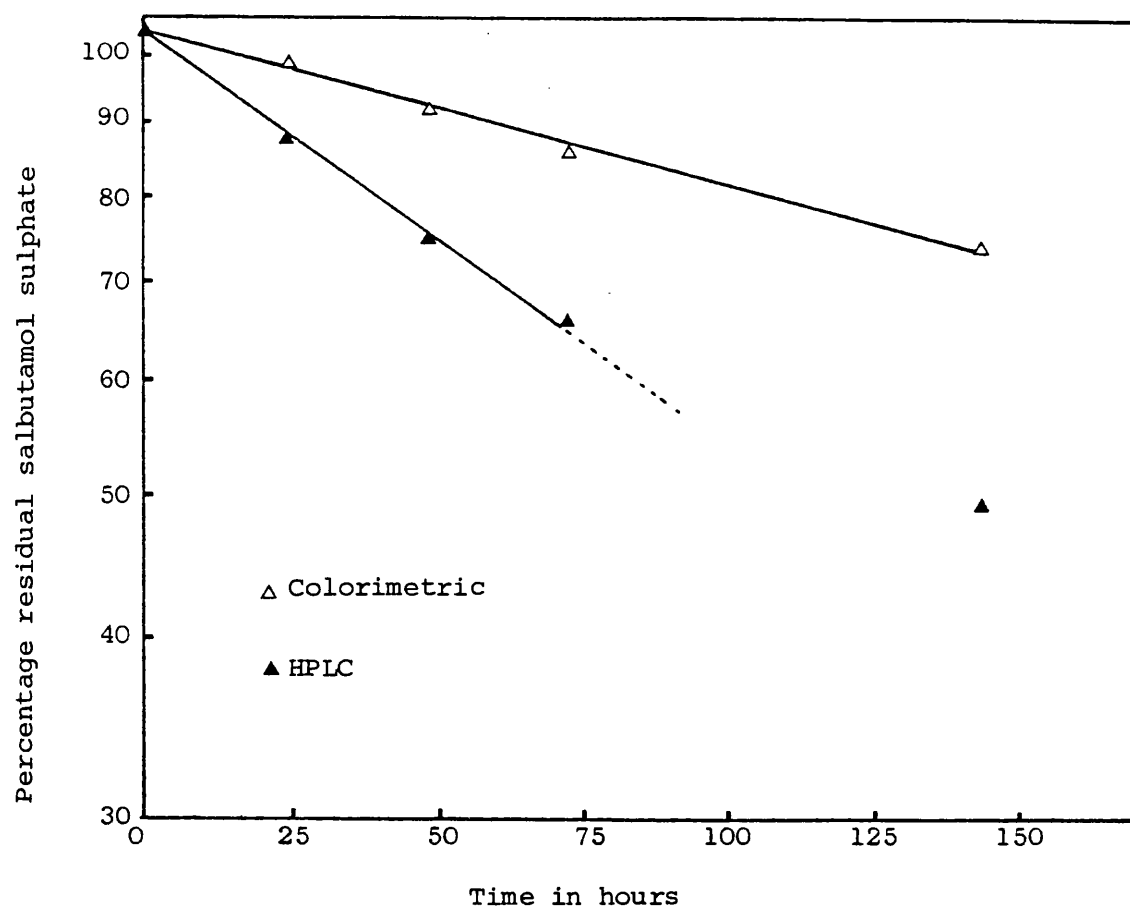


Fig. 25. Comparison of assay methods for degradation of 0.5% Salbutamol sulphate solution at 70° and pH 7.0 in presence of 0.05% EDTA

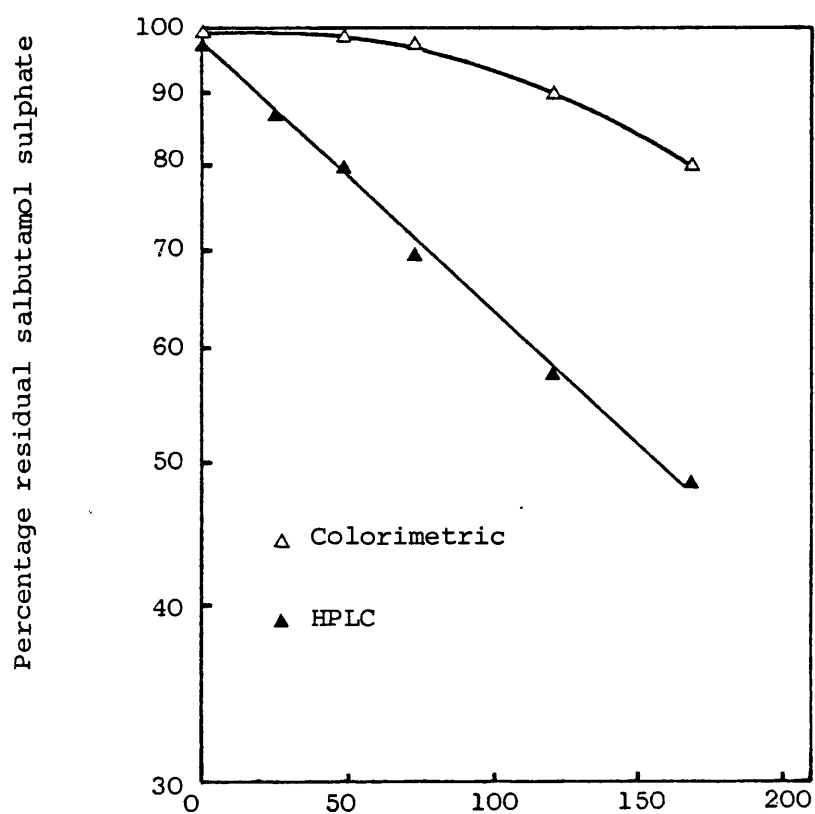


Fig. 26. Comparison of assay methods for the degradation of 0.5% salbutamol sulphate at pH 7.0 and 70°.



| Sample<br>Time (hrs) | Percentage residual salbutamol sulphate |                   |
|----------------------|---|-------------------|
|                      | HPLC Assay                              | Indoaniline assay |
| 0                    | 100.22                                  | 101.31            |
| 24                   | 74.44                                   | 86.95             |
| 48                   | 55.27                                   | 67.69             |
| 71                   | 43.26                                   | 58.88             |
| 108                  | 29.87                                   | 43.86             |

Table 14. Comparison of assay methods for 0.1% salbutamol sulphate solution pH 9, 70<sup>o</sup>, ionic strength 0.5

| Sample (pH<br>adjustment) | Percentage residual salbutamol sulphate |                   |
|---------------------------|---|-------------------|
|                           | HPLC Assay                              | Indoaniline assay |
| Acid                      | 57.36                                   | 69.08             |
| Citrate                   | 76.68                                   | 89.70             |

Table 15. Comparison of assay for industrial stability samples containing 0.05% salbutamol sulphate, pH 3.5 stored under nitrogen for 5 months at 65<sup>o</sup>

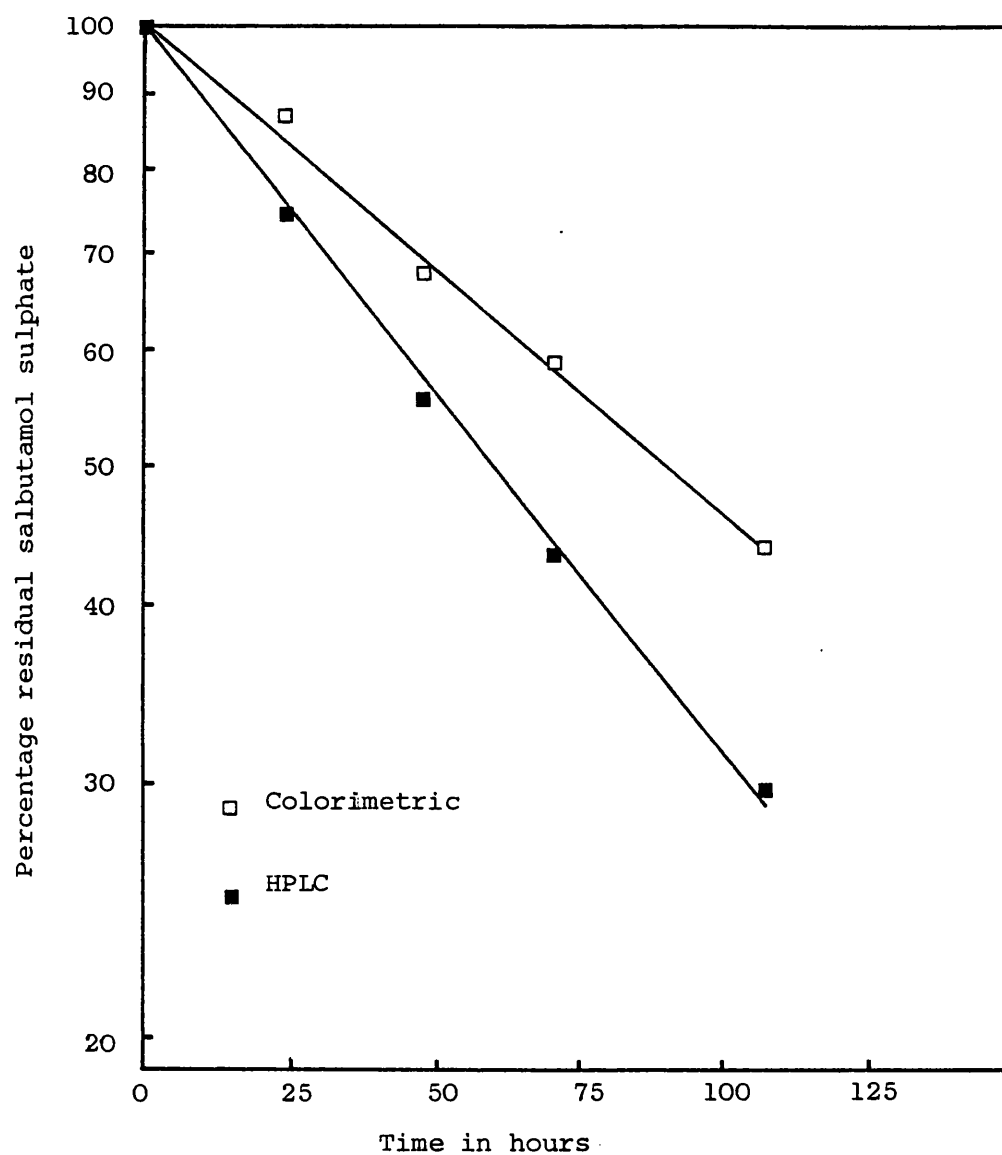


Fig. 27. Comparison of assay methods for the degradation of 0.1% salbutamol sulphate solution containing 0.5M NaCl at pH 9.0 and 70°.

## 2.6 Thin Layer Chromatography

This was used to monitor the pattern of breakdown under various conditions, on a qualitative basis. The plates used were commercially pre-coated with a 250  $\mu\text{m}$  layer of silica gel G and were impregnated with a  $\text{uv}_{254}$  fluorescent indicator. The developing solvent was that used in the B.P. for Salbutamol Sulphate (79) and consisted of ethyl acetate: isopropanol: water:ammonia (0.880), in the ratio 50:30:16:4.

Visualisation was by ultra violet light (254 nm), when spots were visible as dark areas against a green fluorescent background, and by spraying first with MBTH reagent, followed by potassium ferricyanide. The spray reagents were prepared as follows:

a) MBTH Reagent

100 mg MBTH (3-methyl-2-benzothiazide hydrazone hydrochloride  $1\text{H}_2\text{O}$ ) were dissolved in 10 ml water and then made up to 100 ml with methanol

b) Potassium Ferricyanide Reagent

25 ml 0.880 ammonia solution were diluted to 100 ml with water. This solution was used to prepare 2% potassium ferricyanide solution.

After spraying with both reagents, salbutamol and related compounds appear as red spots (Fig. 28). This technique was used to monitor the pattern of salbutamol breakdown in nearly all the kinetic experiments but was found to be sensitive to variations in the laboratory temperature, as demonstrated in Table 16. A standard salbutamol sulphate solution was

therefore run as a standard on each plate and Rf values (Equation 12) could be used only as approximate guides.

$$R_f = \frac{\text{distance moved by solute}}{\text{distance moved by solvent front}} \quad \dots (12)$$



Fig. 28. Photograph of a typical TLC plate, showing the spectrum of breakdown as revealed by the MBTH reagent spray.

therefore run as a standard on each plate and  $R_f$  values (Equation 12) could be used only as approximate guides.

$$R_f = \frac{\text{distance moved by solute}}{\text{distance moved by solvent front}} \quad \dots (12)$$

| Compound                  | $R_f$ |      |
|---------------------------|-------|------|
|                           | 20°   | 24°  |
| Salbutamol                | 0.60  | 0.80 |
| Major degradation product | 0.75  | 0.95 |

Table 16. Changes in  $R_f$  values with temperature for salbutamol and its major degradation product, using the B.P. TLC system (79)

## 2.7 Isolation of Breakdown Products

Attempts were made to isolate and identify the major degradation products of salbutamol in aqueous solution. A combination of the ion-exchange technique developed for the preparation of HPLC samples, and thick layer chromatography was used.

An SP-Sephadex column, 13 cm long and 1.6 cm in diameter was prepared as previously described (Section 2.3). The degraded salbutamol sulphate solution was poured onto the column and allowed to run through the gel. The Sephadex was rinsed thoroughly with several column volumes of distilled water to ensure complete removal of the unretained non-basic compounds. The basic components of the mixture were

eluted, as in the analytical procedure, with dilute ammonia solution.

During the elution with ammonia, two yellow bands were seen to travel down the column. The faster moving band was found, by TLC examination, to consist mainly of salbutamol but the slower band principally comprised degradation products. This band was therefore collected and concentrated on a rotary evaporator until approximately 1 ml solution remained. A steam bath was used as the source of heat during the evaporation and care was taken to ensure that the flask and its contents never became warm to the touch. In this way, further degradation should have been minimised.

The concentrated solution was applied as a streak to 1000  $\mu\text{m}$  thick silica gel G preparative TLC plates, containing a  $\text{uv}_{254}$  fluorescent indicator. The plate was allowed to dry thoroughly, usually by leaving it overnight, before developing it using the TLC solvent system. A continuous elution tank was used for the thick layer plates since  $R_f$  values tended to be lower than on thin layer plates. Development was allowed to continue for approximately  $3\frac{1}{2}$  hours before removing the plate and allowing it to dry.

The developed plate was examined under ultraviolet light (254 nm) when the bands of the components were visible as dark lines against a fluorescent background. The position of the band of interest was marked and the appropriate area was then carefully scraped off the glass and the silica

collected in a glass chromatography column. The adsorbed compounds were eluted with methanol and this methanolic solution was passed through an SP-Sephadex column, washed with water and eluted with ammonia as before. This was necessary to remove acetamide, formed by the TLC solvent and therefore present as a contaminant.

The ammoniacal solution was finally evaporated to dryness on a rotary evaporator using a minimum amount of heat. The resultant solid was examined by various spectrographic techniques, including proton and carbon-13 nuclear magnetic resonance (nmr) and mass spectrometry.

## B. KINETIC STUDIES

### Materials

Salbutamol sulphate was B.P. grade and was used as supplied by Allen and Hanburys Research Ltd. (now Glaxo Group Research), without further purification. A single batch was used throughout the work.

Buffer salts were of AnalaR quality.

Sodium hydroxide for the pH-stat was of AnalaR quality.

Glucose and sucrose were AnalaR grade (BDH Chemicals Ltd.).

Glycerin and propylene glycol were B.P. grade (Evans Ltd.).

Fructose was laboratory reagent grade and "glucose free" (BDH Chemicals Ltd.).

Glyceric acid was obtained as a 65% solution from Aldrich Chemicals.

Glyceraldehyde was laboratory reagent grade (BDH Chemicals)

5-hydroxymethyl furfural was obtained from Sigma Ltd.

Sorbitol was obtained as a 70% solution from Atlas Chemicals.

Oxygen and "high purity" nitrogen were obtained in cylinders from Air Products Ltd.

Water was freshly distilled from an all glass still.

Ingold electrode cleaner solutions 9891 and 9892 (Pye Unicam Ltd.)

### Equipment

Water baths were heated and thermostatically controlled to within  $\pm 0.05^{\circ}$  by Grant SU6 water bath heaters.

Three sets of pH-stat equipment were used:



- a) Radiometer Titrator 11  
Radiometer Autoburette ABU 12  
Radiometer pH meter 26
- b) Radiometer Titrator TTT60  
Radiometer Autoburette ABU 12  
Radiometer pH meter pHM61
- c) Radiometer Titrator TTT60  
Radiometer Autoburette ABU 12  
Radiometer pH meter pHM64

Pye Ingold high temperature electrodes (465-90) were used as sensors for each unit.

Radiometer glass burettes and pistons of 0.25 ml and 2.5 ml capacity were used to deliver the pH-stat titrant.

## 2.8 pH-statting

Most published stability studies have been performed using buffer salts to control the pH of the system in spite of the fact that buffers can affect the rate of reaction (10, 42, 44-46). Such an approach therefore necessitates a study of the size and nature of such effects so that due allowance can be made for them in assessing the results of stability experiments.

These problems are avoided by the use of pH-statting techniques and the interpretation of the results is therefore simpler. The effects of buffer salts may then be studied separately once the "base-rate" of reaction has been determined. The pH is controlled by automatic titration equipment which monitors the pH and delivers small volumes of a suitable titrant to correct any changes.

Three different sets of equipment were used in these studies, each consisting of a pH-meter, a high temperature electrode, an electronic titration control unit and an automatic burette. In each case the manufacturer's specification of pH control to within  $\pm 0.05$  pH units was determined by the operational limits of the titrator unit involved. The TTT60 instruments have now replaced the older titrator 11 units in the Radiometer range.

Although the pH meters have different specifications, all were capable of an accuracy of 0.03 pH units or better. The pH meter 26 which interfaced with the titrator 11 unit

is now obsolete but the equipment is still functioning perfectly. The pH range of pH meters are necessary to interface with the TTT60 titrators. The specification of the pHM61, a pointer-scale instrument, is adequate for the titrator; the digital pHM64 is a technically superior instrument but since the titrator performance is the limiting factor, this instrument has no advantage over the pHM61 in pH statting units. It was used in this study because of its availability within the laboratory.

Pye-Ingold 465-90 high temperature dual electrodes were used because they are designed to operate continuously for long periods at high temperature. More conventional electrodes, such as the Radiometer GK2401C, although capable of sensing pH at temperatures up to about  $70^{\circ}$ , can only be operated for relatively short periods at elevated temperatures.

Prolonged use of the electrodes can result in contamination of the sintered plug linking the glass and reference electrode compartments. This causes loss of sensitivity and a sluggish response and so sensitivity and response rate were checked before and after each experiment, using standard buffers (105). The response drift was always less than 0.05 pH units. A typical set of results, following the degradation of 0.5% salbutamol sulphate in 50% glucose solution at pH 3.5 and  $70^{\circ}$  for 500 hours, gave an electrode response of 4.15 for standard phthalate buffer (pH 4.12 at  $70^{\circ}$ ) and 1.67 for standard tetroxalate buffer (pH 1.68 at  $70^{\circ}$ ). As a precaution, the electrodes were cleaned at

intervals with Ingold cleaner solutions 9891 and 9892 according to the protocol given by the manufacturer.

The ABU12 autoburettes provide a piston drive to glass burettes with capacities of 0.25, 2.5 or 25 ml. These instruments can be operated in either continuous or pre-determined incremental delivery volume modes. The former was used in this work, allowing smooth addition of titrant on activation by the titrator unit. The speed of titration could be varied from 40 seconds to 80 minutes for full burette delivery when operated without interruption.

The titrator units are the control modules of the pH-statting system. They receive the voltage from the pH meter which changes as the pH alters. When the input voltage moves outside the range corresponding to the preset pH (e.g.  $\text{pH} \pm 0.05$ ), the titrator activates the autoburette which then adds titrant until the voltage, and hence the pH, is restored to the predetermined value.

It is therefore important to be able to control the rate of titrant delivery so that the pH does not deviate significantly from the preset value. If the delivery rate is too high, the titrant too strong, the titrator sensitivity too low or any combination of these, then the pH correction will overshoot as indicated diagrammatically in Fig. 29a.

If the converse situation occurs and insufficient titrant is added, the pH change may outpace the titrant additions and

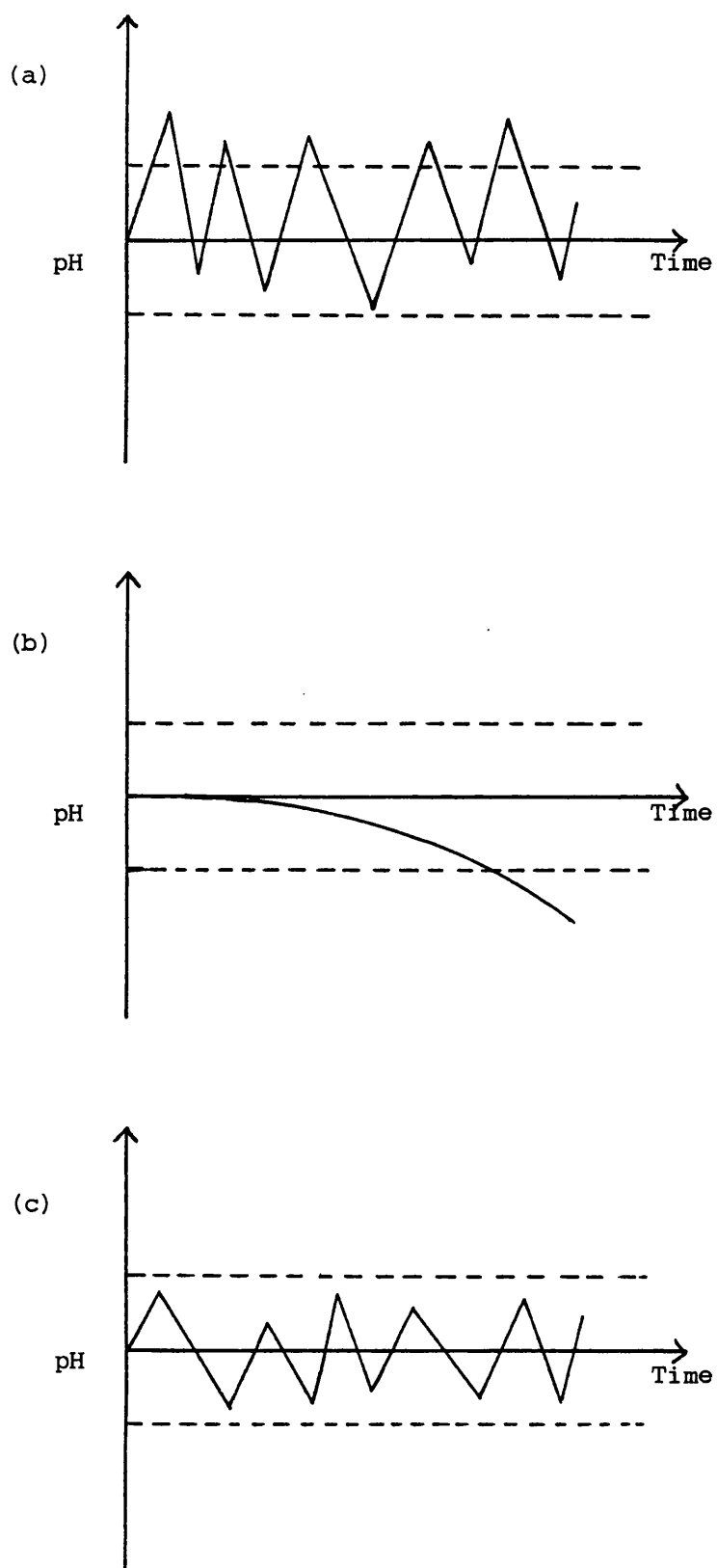


Fig. 29. Schematic representation of pH control in pH-stat

- (a) Titrant addition too fast or too strong
- (b) Titrant addition too slow or too weak
- (c) Ideal control.

so drift away from the required value (Fig. 29b).

A judicious combination of titrator sensitivity, burette speed, titrant strength and burette size is therefore necessary to maintain the pH within the desired limits (Fig. 29c). This combination will vary according to the nature of the reaction mixture and must be determined by preliminary experimentation for each set of conditions. The pH variation during statting can be monitored on the pH meter and was never more than  $\pm 0.05$  pH units in any experiment.

## 2.9 General Method

A photograph of the apparatus used is presented in Fig. 30 and the system is represented diagrammatically in Fig. 31.

The pH electrodes were cleaned by soaking in electrode cleaner solution No. 9891 followed by solution No. 9892 for at least three hours each, rinsing well afterwards with distilled water. The electrodes were then standardised using buffer solutions equilibrated to the temperature of the experiment. The buffers used were 0.05M potassium hydrogen phthalate and 0.01M sodium borate (105) for experiments at pH 4 or higher and 0.05M potassium tetroxalate (105) for experiments below pH 4. Buffer solutions were stored in a refrigerator for no longer than one month.

The large bulbous reservoir of the high temperature electrodes has a high surface area which can cause appreciable

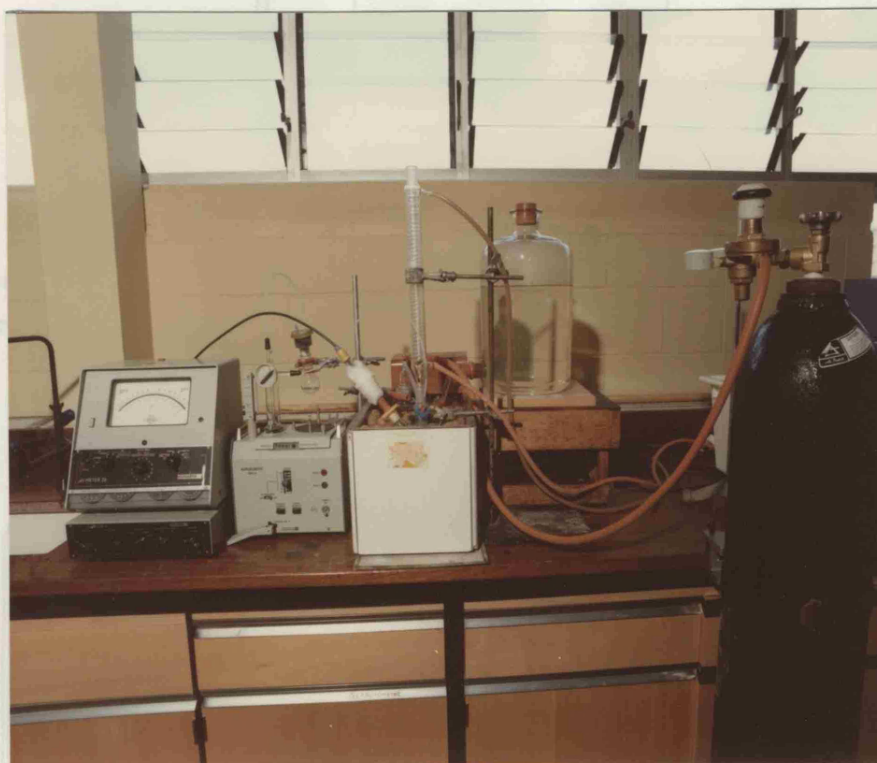


Fig. 30. Photograph of the pH-stat equipment used for the kinetic experiments on salbutamol sulphate degradation.

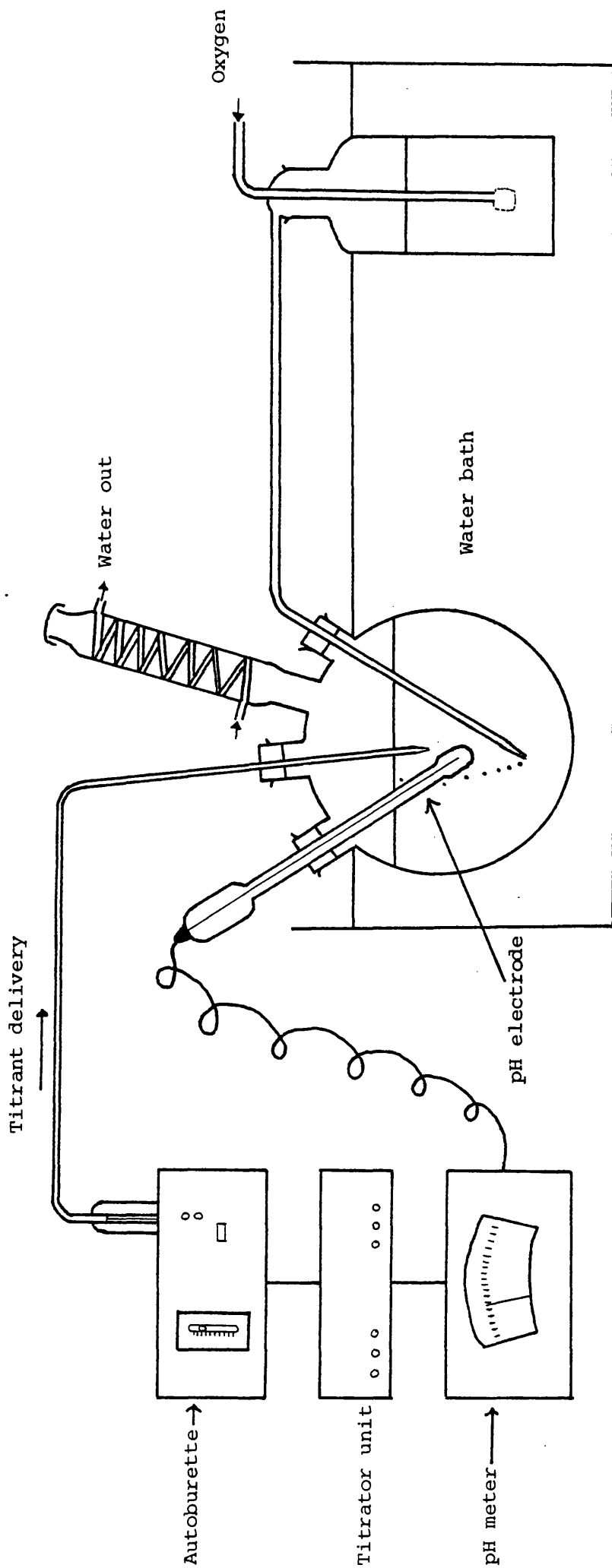


Fig. 31. Diagrammatic representation of pH-stat apparatus.



heat losses. These would result in a temperature gradient along the electrode leading to errors in electrode response. The portion of each electrode outside the reaction vessel was therefore lagged with a 1 cm layer of cellulose wadding to minimise heat losses and stabilise the instrument response.

The reaction vessel consisted of a 100 ml or 250 ml Quickfit flask with four necks. During an experiment, a single coil reflux condenser was fitted to the central neck. The other three were used for the pH electrode, the oxygen inlet and the pH-stat titrant inlet. Humidified oxygen was bubbled through the experimental solutions at a rate of 10 - 15 ml per minute via a narrow glass tube. There proved to be insufficient space within the flask to enable a sintered glass oxygen inlet to be used. The oxygen flow provided sufficient agitation to ensure thorough mixing of the solution without the need for a mechanical stirrer.

The titrant was delivered from the autoburette via narrow bore polythene tubing (I.D. 2 mm) with a glass tip which dipped into the reaction mixture. The glass tip was drawn out into a fine capillary which admitted the titrant to the reaction vessel in a very thin stream. This was rapidly dispersed throughout the solution and so minimised the local development of a high pH around the tip. The tip was also positioned close to the pH electrode bulb (but not touching it) in order to minimise the time lag between titrant addition and electrode response and thus minimise

the chance of "pH overshoot". The solution was sampled by temporarily removing the oxygen inlet and withdrawing an aliquot by pipette.

For kinetic studies on simple salbutamol sulphate solutions, 75 ml or 175 ml water, as appropriate for the flask size, were pipetted into the flask. This was clamped in the water bath and allowed to equilibrate for one hour, during which time humidified oxygen was bubbled through the liquid. The appropriate weight of drug was added as 10 ml concentrated solution from a 10 ml volumetric flask. This was rinsed with measured volumes of distilled water to bring the total volume of solution in the reaction vessel to 90 ml or 200 ml. This procedure was adopted to avoid the need to store concentrated stock solutions of the drug which may have proved unstable. The temperature change on the addition of the drug was negligible, equilibrium being reached within two minutes. The pH was adjusted to the required value with sodium hydroxide solution or sulphuric acid and an initial sample was withdrawn. Further samples were withdrawn at suitable intervals, rapidly cooled and stored in a refrigerator for no longer than three days until required for assay. Samples which were assayed immediately and again after seven days gave consistent results as demonstrated in Table 17.

In experiments designed to study the effect of other compounds on the breakdown of salbutamol sulphate, the additive was dissolved in the appropriate volume of water and equilibrated in the reaction vessel before the addition

| Sample                       | % salbutamol sulphate<br>remaining (immediate<br>assay) | % salbutamol sulphate<br>remaining (delayed assay) |
|------------------------------|---|--|
| pH 5 initial                 | 101.97 ( $\pm 0.51$ )                                   | 100.89 ( $\pm 0.79$ )                              |
| pH 9 42 hrs                  | 36.99 ( $\pm 0.28$ )                                    | 37.24 ( $\pm 0.36$ )                               |
| 50% glucose<br>pH 3.5 246hrs | 68.53 ( $\pm 0.35$ )                                    | 68.62 ( $\pm 0.29$ )                               |

Table 17. Stability of experimental samples on storage in a refrigerator for seven days (figures in brackets are standard deviations).

of the drug. In all other respects the experiments were performed in the same manner as those on simple salbutamol sulphate solutions.

The pH-stat titrant was sodium hydroxide solution, since the pH always fell as the drug degraded. The strength of the titrant was such that the total volume added to the flask during an experiment was no more than 1% of the flask contents. Concentrations of approximately 1.0 M to 5.0 M were usually needed. The settings of the titrator controls were chosen to ensure that the pH of the reaction solution was maintained within 0.05 pH units of the selected value. The delayed mixing in the viscous 50% sugar solutions necessitated slower titrant addition to prevent overshooting the required pH.

## 2.10 Treatment of Results

The residual concentration of salbutamol sulphate was

calculated as a percentage of the theoretical initial concentration (Section 2.3, Table 7) and the data plotted against time according to first order kinetics. If the graph indicated a fairly good linear relationship, the results were statistically assessed using least squares regression analysis (Appendix 1) to give an apparent first order rate constant. When the first order plot was not linear over the full course of the reaction, an initial apparent first order rate constant was calculated by applying least squares regression analysis to the initial, linear, data points as illustrated in Fig. 32. When this method has been adopted, the number of data points,  $n$ , used in the rate constant determination is indicated.

## 2.11 Reproducibility of Results

The stability of a 2.0% salbutamol sulphate solution at pH 9.0 and 70° with an oxygen flow rate of 10 - 15 ml per minute, was determined on three separate occasions over the course of one month and the data obtained are presented in Table 18 and Fig. 33. The first order rate constants are also presented in the table and these were subjected to a Bartlett test (Appendix 1). The calculated value of  $\chi^2$  was 0.866 and the tabulated value at the 5% probability level is 5.991. The rate constants are therefore not significantly different and the experimental method was considered to be reproducible.

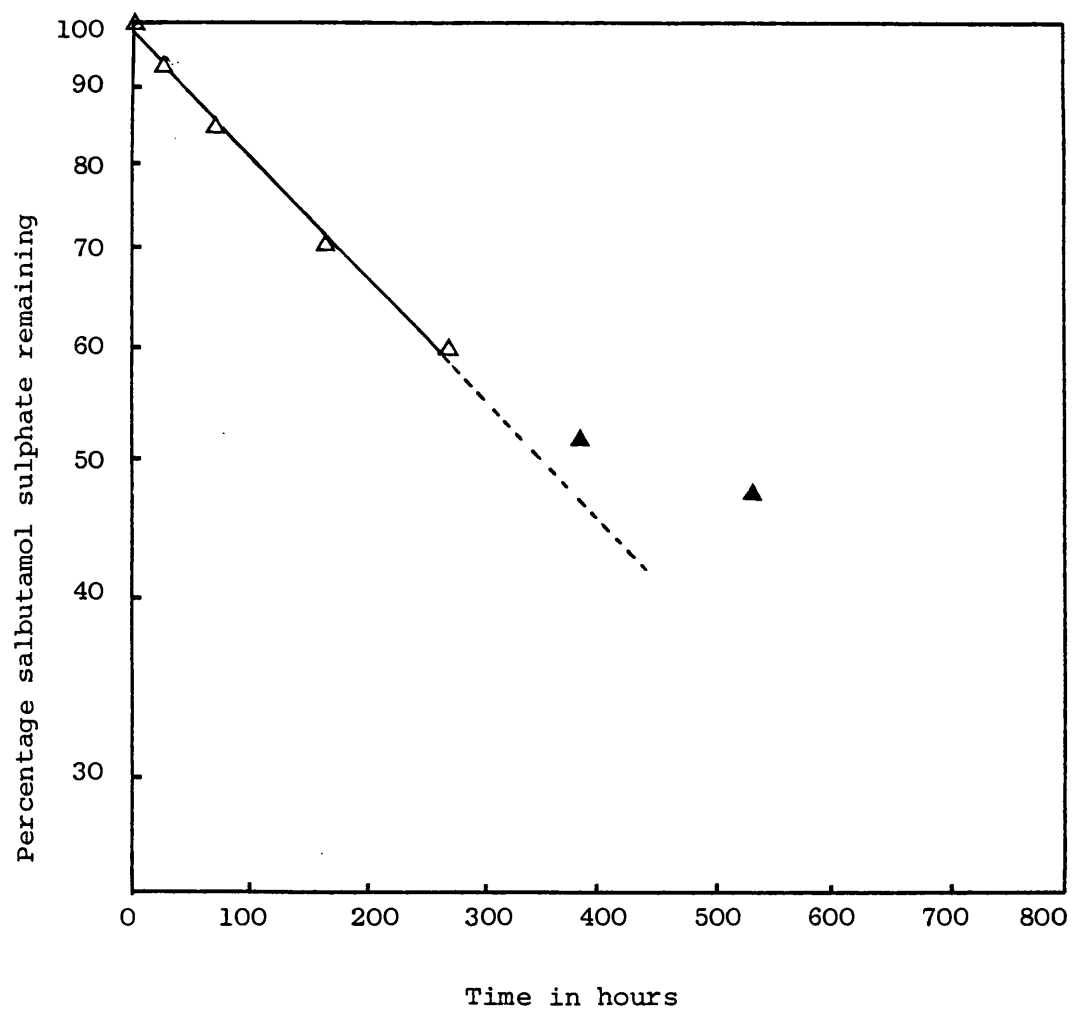


Fig. 32. Treatment of non-linear results. The open symbols represent points used in the calculation of the apparent first order rate constant.

| Time in<br>hours | % Salbutamol sulphate<br>remaining | Time in<br>hours | % Salbutamol sulphate<br>remaining | Time in<br>hours | % Salbutamol sulphate<br>remaining |
|------------------|------------------------------------|------------------|------------------------------------|------------------|------------------------------------|
| 0                | 96.74                              | 0                | 93.46                              | 0                | 91.53                              |
| 1                | 95.85                              | 4                | 82.05                              | 3.5              | 78.66                              |
| 2                | 94.44                              | 10               | 65.00                              | 11.25            | 59.51                              |
| 5                | 88.22                              | 18               | 48.10                              | 19.5             | 42.06                              |
| 8                | 73.37                              | 26.5             | 35.85                              | 27.5             | 32.90                              |
| 12               | 63.00                              | 34               | 27.15                              | 33.5             | 28.58                              |
| 18               | 50.29                              |                  |                                    |                  |                                    |
| 23               | 42.46                              |                  |                                    |                  |                                    |
| 29               | 35.24                              |                  |                                    |                  |                                    |
| 33.5             | 30.26                              |                  |                                    |                  |                                    |
| 39               | 25.38                              |                  |                                    |                  |                                    |

|  |  |  |
|--|--|--|
| $k = 9.89 \times 10^{-6} \text{ s}^{-1}$ | $k = 1.01 \times 10^{-5} \text{ s}^{-1}$ | $k = 9.83 \times 10^{-6} \text{ s}^{-1}$ |
| Std.Dev. = $1.98 \times 10^{-7}$         | Std.Dev. = $8.31 \times 10^{-8}$         | Std.Dev. = $3.97 \times 10^{-7}$         |
| $r = 0.9982$                             | $r = 0.9999$                             | $r = 0.9967$                             |

Table 18. Data for the stability of 2.0% salbutamol sulphate solution at pH 9 and 70° including apparent first order rate constants (k) (r = correlation coefficient).

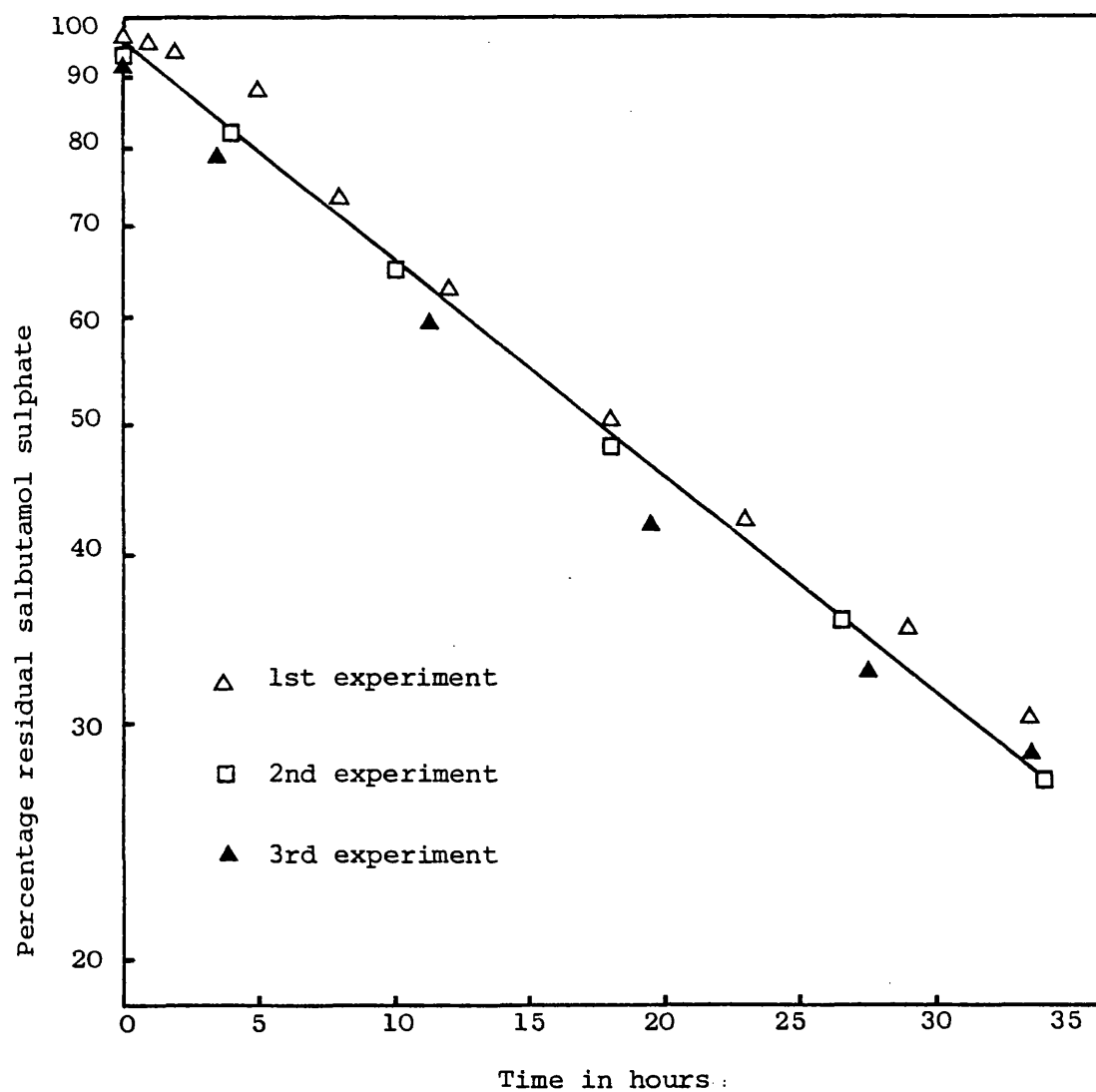


Fig. 33. Reproducibility of kinetic results. Degradation of 2.0% salbutamol sulphate solutions at 70° and pH 9.0.

## 2.12 Stability Studies

Most experiments were performed using the general pH-statting technique described in Section 2.9. Different techniques were adopted for a few experiments and these are described at the relevant point.

### a) The effect of oxygen

Preliminary experiments carried out with 2% salbutamol sulphate solutions at pH 9.0 and 70° showed that when the solution was open to the atmosphere via the condenser and mechanically stirred with an underwater magnetic stirrer, the drug degradation pattern was not reproducible and the first order plots were non-linear (Figure 34). Oxygen starvation was considered to be the most likely cause of these effects. The amount of air drawn into the solution by the stirring action would be dependent on the stirrer speed and gaseous equilibration is probably slow relative to the rate of oxygen utilisation during the degradative processes. Consequently, a fine stream of humidified gas was bubbled through experimental solutions via a narrow glass tube. This not only provided a more efficient gas-liquid interface but also ensured thorough mixing of the solution (Section 2.9).

The effect of oxygenation was studied by bubbling solutions containing 0.5% salbutamol sulphate at pH 9.0 and 70°, with oxygen at 10 ml per minute and 50 ml per minute and also with white spot nitrogen at 20 ml per minute. The



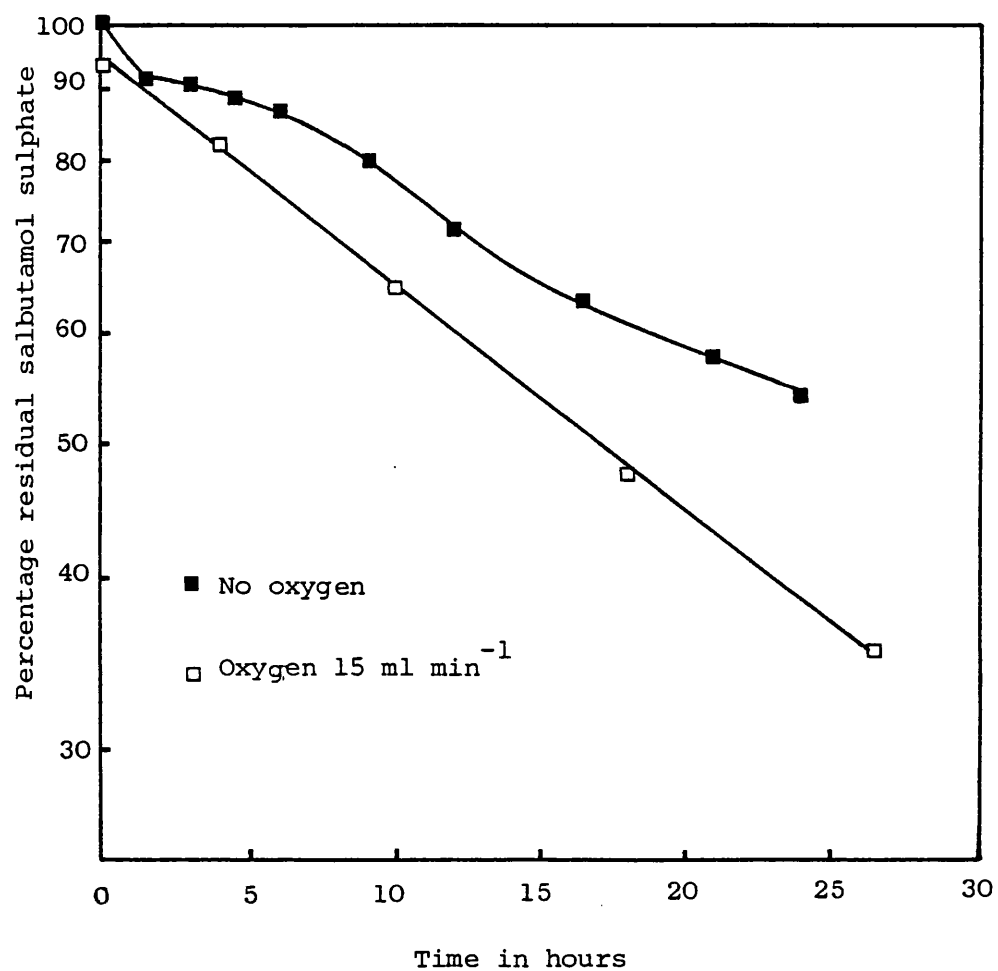


Fig. 34. The degradation of 2.0% salbutamol sulphate solution in the presence and absence of bubbled oxygen.

data from the three experiments are presented in Table 19 and Figure 35.

The results indicate that under oxygen the degradation rate is almost three times greater than that under nitrogen. The graph also suggests that an increase in oxygen flow rate from 10 to 50 ml per minute results in no change in degradation rate. A "t" test (Appendix 1) between the apparent first order rate constants under oxygen gave a t value of 1.950 compared with a tabulated value of 2.306 at the 5% probability level, indicating no significant difference between the rate constants. An oxygen flow rate of 10 - 15 ml per minute was therefore used in all subsequent experiments.

b) The effect of drug concentration

Experiments were conducted at 70° and pH 9.0 using salbutamol sulphate concentrations of 0.1%, 0.5%, 1.0%, 2.0%. Linear graphs were obtained when the results were plotted according to first order kinetics (Fig. 36) but the apparent first order rate constants increased with concentration (Table 20), indicating that the breakdown of salbutamol sulphate is not a true first order process.

As the degradation rate was shown to be independent of ionic strength (Section 2.12c), the half-life of the drug at each concentration was calculated from the appropriate rate constant (Table 20). The logarithm of the half-life

| Oxygen (10 ml min <sup>-1</sup> )        |                                 | Oxygen (50ml min <sup>-1</sup> )         |                                 | Nitrogen (20mlmin <sup>-1</sup> )        |                                 |
|--|---------------------------------|--|---------------------------------|--|---------------------------------|
| Time (hours)                             | % Salbutamol sulphate remaining | Time (hrs)                               | % Salbutamol sulphate remaining | Time (hrs)                               | % Salbutamol sulphate remaining |
| 0  | 99.54                           | 0  | 98.89                           | 0  | 96.70                           |
| 3  | 92.51                           | 3  | 93.23                           | 24                                       | 78.60                           |
| 9  | 77.78                           | 11                                       | 75.01                           | 49                                       | 61.71                           |
| 17                                       | 64.06                           | 21                                       | 58.65                           | 73                                       | 51.21                           |
| 29                                       | 47.35                           | 34                                       | 42.90                           | 115.5                                    | 35.01                           |
| 43                                       | 33.73                           | 53                                       | 27.26                           |  |                                 |
| $k = 7.00 \times 10^{-6} \text{ s}^{-1}$ |                                 | $k = 6.81 \times 10^{-6} \text{ s}^{-1}$ |                                 | $k = 2.44 \times 10^{-6} \text{ s}^{-1}$ |                                 |
| Std.Dev. = $7.78 \times 10^{-8}$         |                                 | Std.Dev. = $6.22 \times 10^{-8}$         |                                 | Std.Dev. = $3.47 \times 10^{-8}$         |                                 |

Table 19. The degradation of 0.5% salbutamol sulphate solution at pH 9 and 70° under aerobic and anaerobic conditions

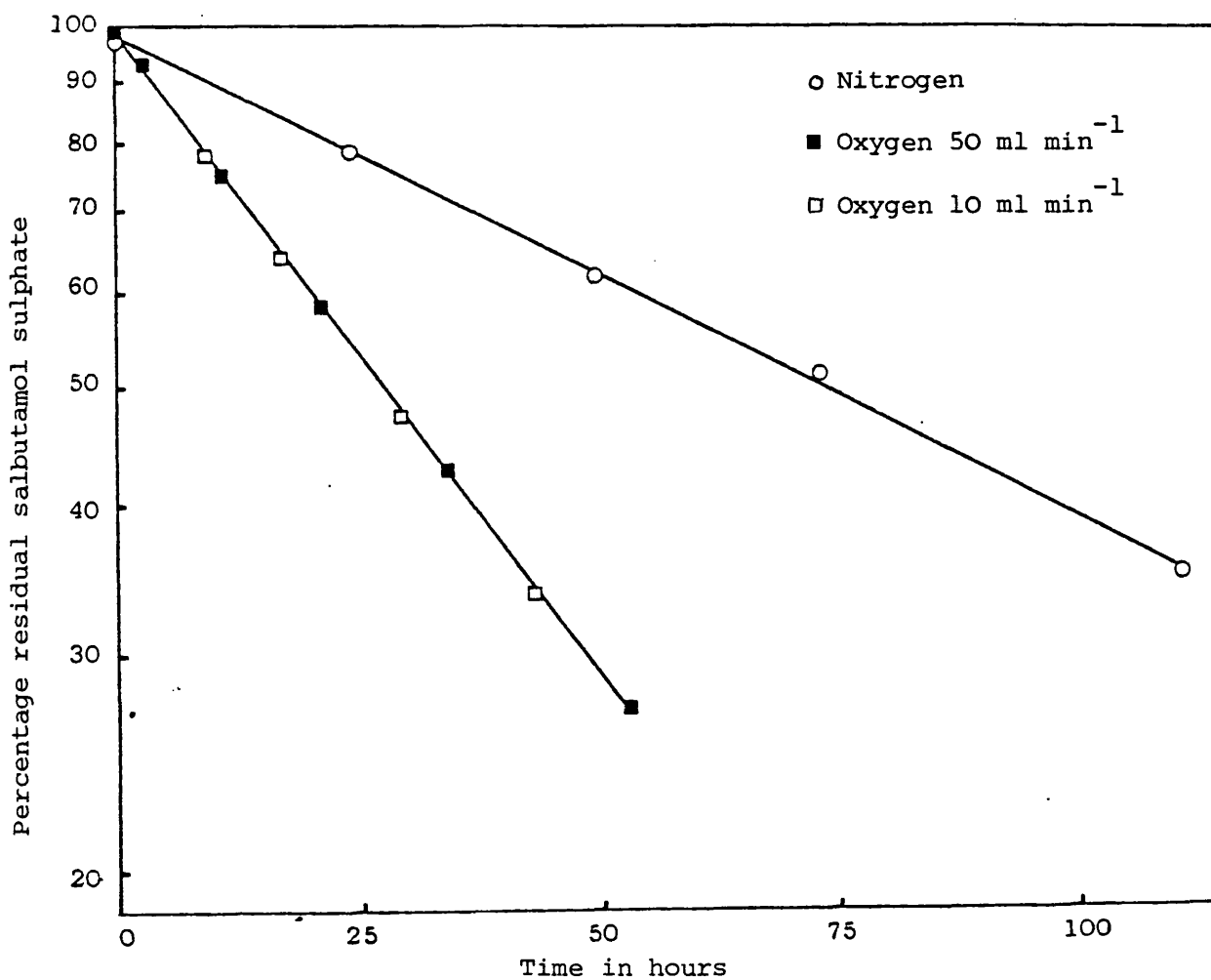


Fig. 35. The effect of oxygen on the degradation of 0.5% salbutamol sulphate solution at pH 7.0 and 70°.

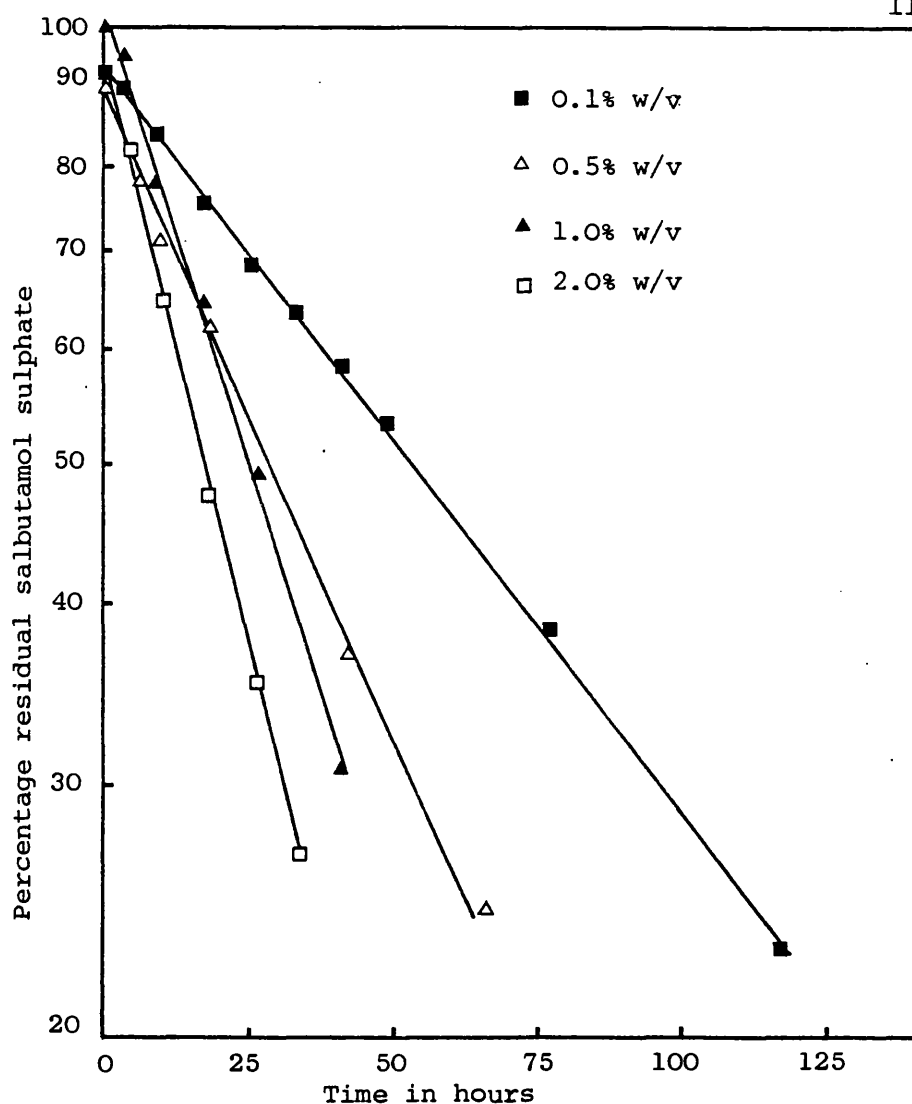


Fig. 36. The effect of drug concentration on the degradation of salbutamol sulphate at pH 9.0 and 70°.

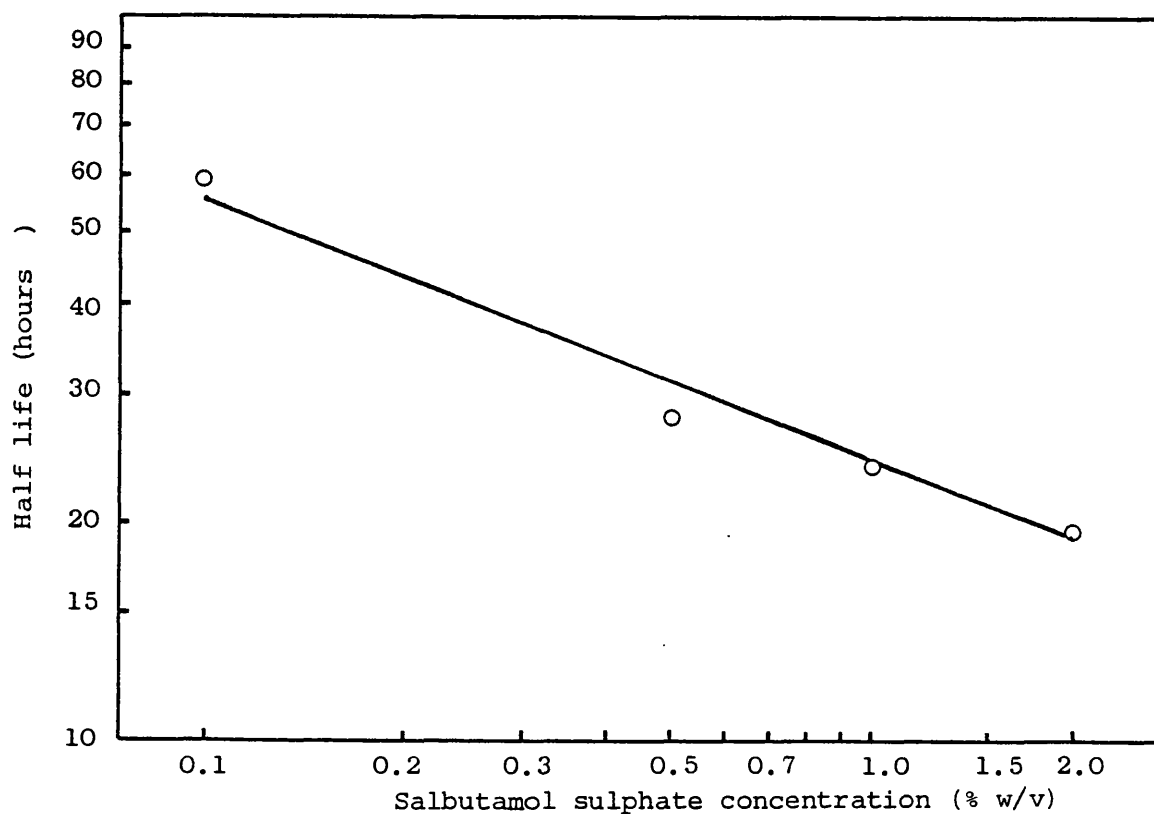


Fig. 37. Half-life plot for determination of order of reaction for salbutamol sulphate degradation at pH 9.0 and 70°.

was plotted against the logarithm of the concentration, according to the half-life method for determination of the order of reaction (Section 1.3). The result is shown in Figure 37, the line of which has a slope of -0.375 with a standard deviation of 0.040. The order of reaction is therefore approximately 1.4 (Equation 7)

| Salbutamol sulphate<br>concentration (%<br>w/v) | Rate constant<br>$k, (s^{-1}) \times 10^6$ | Std.Dev.<br>$\times 10^7$ | Half Life,<br>$t_{1/2}$<br>(hrs) | Mean<br>$t_{1/2}$<br>(hrs) |
|---|--|---------------------------|----------------------------------|----------------------------|
| 0.1   | 3.25                                       | 0.333                     | 59.23                            | 59.23                      |
| 0.5   | 7.00<br>6.81                               | 0.778<br>0.622            | 27.50<br>28.29                   | 27.90                      |
| 1.0   | 8.03                                       | 2.49                      | 23.98                            | 23.98                      |
| 2.0   | 9.89<br>10.14<br>9.83                      | 1.98<br>0.831<br>3.97     | 19.47<br>18.99<br>19.58          | 19.35                      |

Table 20. Data showing the effect of drug concentration on the apparent first order rate constant and half-life for the degradation of salbutamol sulphate at pH 9 and 70°

c) The effect of ionic strength

The change in the rate constant with concentration could be due to ionic strength effects as the ionic strength will vary by a factor of 20 over the concentration range 0.1% to 2.0% salbutamol sulphate. The stability of a 0.1% salbutamol sulphate solution in the presence of 0.5M sodium chloride at pH 9 and 70° was therefore examined. This concentration

of added electrolyte will produce an ionic strength of approximately 0.5M which is greatly in excess of that of a 2.0% salbutamol sulphate solution (approximately 0.06M). The result is presented in Table 21, together with that for the solution with no ionic strength adjustment, and is depicted in Figure 38.

| Added NaCl | $k(s^{-1}) \times 10^6$ | Std.Dev. $\times 10^8$ |
|------------|-------------------------|------------------------|
| None       | 3.25                    | 3.33                   |
| 0.5M       | 3.08                    | 8.33                   |

Table 21. The effect of ionic strength on the apparent first order rate constant for the degradation of 0.1% salbutamol sulphate solution at pH 9 and 70°

The rate constants were compared using a Student's t-test (Appendix 1) which gave a t value of 1.999. The tabulated value at the 5% probability level and 13 degrees of freedom is 2.160 and so the rate constants cannot be considered significantly different. Since ionic strength had no effect on the rate of degradation, it was not adjusted in subsequent experiments.

d) The effect of temperature

The effect of temperature on the rate of breakdown of salbutamol sulphate was examined at both pH 9.0 and pH 7.0, using a drug concentration of 0.5%. At pH 9.0, the

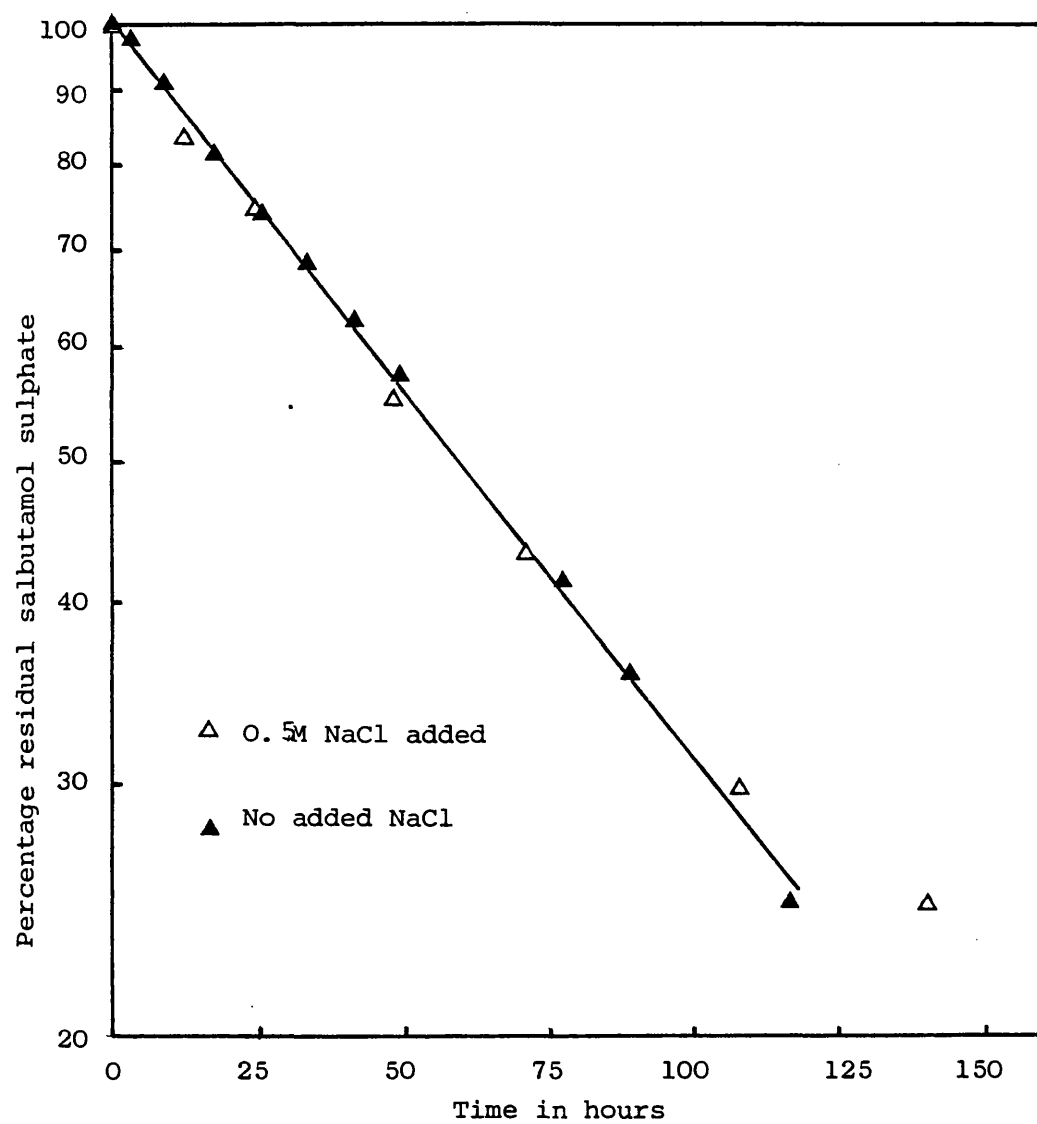


Fig. 38. The effect of ionic strength on the degradation of 0.1% salbutamol sulphate solution at pH 9.0 and 70°.

temperatures studied were 50°, 55°, 60°, 70° and 75°, whilst at pH 7.0 the range was from 60° to 85° in 5° increments. The temperature was monitored during each experiment using a Zeal thermometer calibrated to 0.1° and was found to vary by no more than  $\pm 0.1^\circ$ . The apparent first order rate constant was determined for each set of conditions (Table 22) and the values obtained plotted according to the Arrhenius relationship (Fig. 39). These lines were subjected to least squares regression analysis (Appendix 1) from which the activation energy for the degradative process at each pH could be calculated (Table 23).

| Temperature | k (pH9)<br>$s^{-1} \times 10^6$ | Std.Dev.<br>$\times 10^8$ | k (pH7)<br>$s^{-1} \times 10^6$ | Std.Dev.<br>$\times 10^8$ |
|-------------|---------------------------------|---------------------------|---------------------------------|---------------------------|
| 50°         | 0.636                           | 2.49                      |                                 |                           |
| 55°         | 1.17                            | 1.36                      |                                 |                           |
| 60°         | 2.03                            | 2.81                      | 0.328                           | 1.06                      |
| 65°         |                                 |                           | 0.669                           | 1.44                      |
| 70°         | 7.00                            | 7.78                      | 1.08                            | 3.92                      |
| 75°         | 10.4                            | 12.0                      | 1.48                            | 5.72                      |
| 80°         |                                 |                           | 3.03                            | 7.22                      |
| 85°         |                                 |                           | 5.53                            | 9.17                      |

Table 22. The effect of temperature on the apparent first order rate constant for the degradation of 0.5% salbutamol sulphate solution at pH 9.0 and pH 7.0.



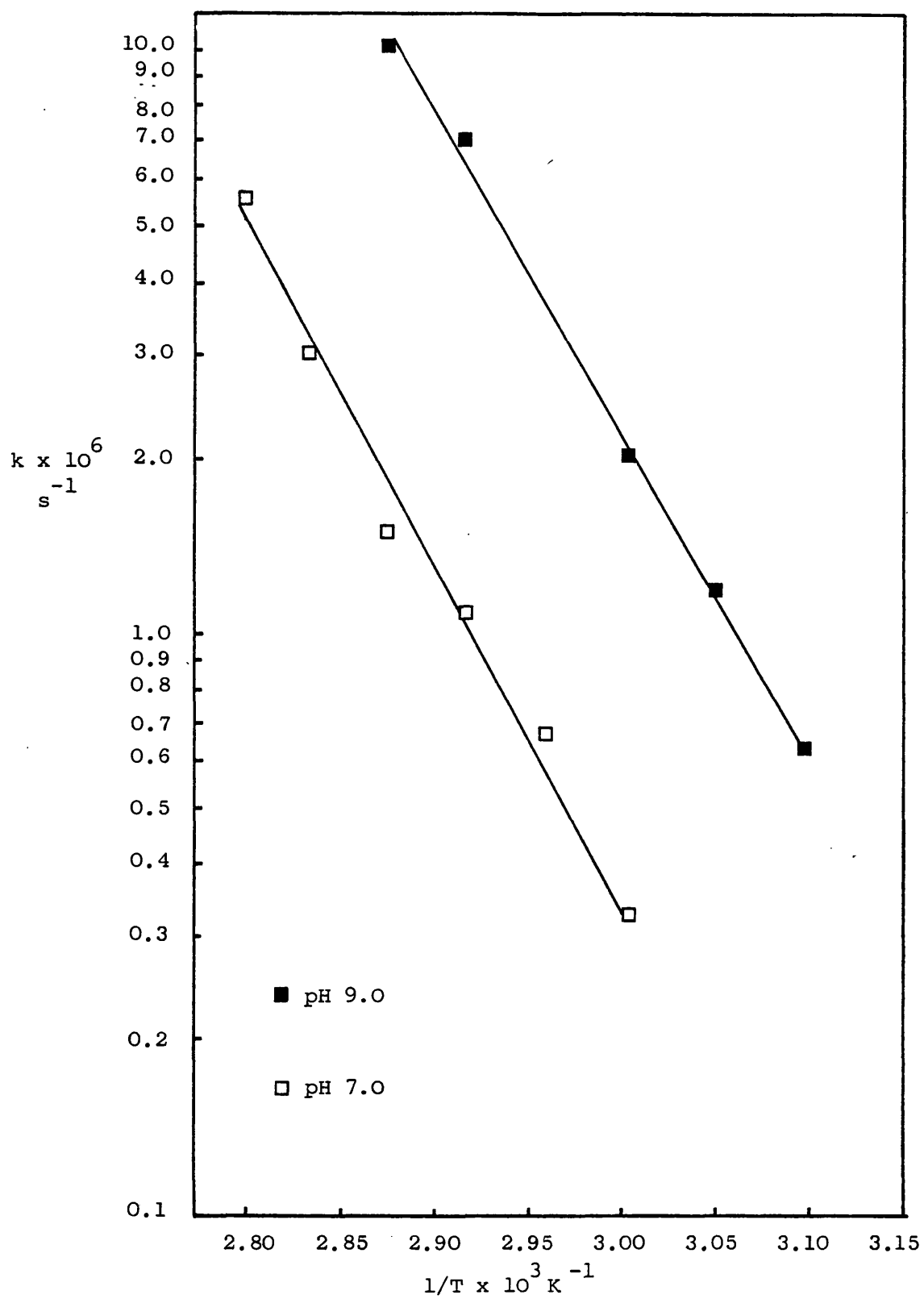


Fig. 39. Arrhenius plots for the degradation of 0.5% salbutamol sulphate at pH 9 and pH 7.

| pH | Slope               | Std.Dev.           | Intercept | Std. Dev. | Correlation coefficient | Activation energy (KJmol <sup>-1</sup> ) | Std. Dev. |
|----|---------------------|--------------------|-----------|-----------|-------------------------|--|-----------|
| 9  | $-5.58 \times 10^3$ | $1.49 \times 10^2$ | 11.07     | 0.44      | 0.999                   | 106.84                                   | 2.85      |
| 7  | $-5.60 \times 10^3$ | $2.98 \times 10^2$ | 10.35     | 0.30      | 0.994                   | 107.22                                   | 5.71      |

Activation energy = - slope x 8.314 x 2.303

Table 23. Data obtained from the Arrhenius graphs (Fig. 39) for the degradation of 0.5% salbutamol sulphate solutions at pH 9 and pH 7.

The activation energies were subjected to a Student's t-test which gave a t value of 0.06. The tabulated value at the 5% probability level is 2.365 and so the activation energies are not significantly different. This indicates that there is no change in the reaction mechanism between pH7.0 and pH9.0.

e) The effect of pH

A pH-stability profile of the drug at 70° was generated over the pH range 1 - 10, using a salbutamol sulphate concentration of 0.5%. It was found that below pH 4, the solutions were self-buffering, rendering the pH-stat unnecessary. The rate of breakdown was also low in the region pH 2 - 5 and so the solutions were kept in stoppered flasks and bubbled with oxygen at regular intervals. This allowed the pH-stat equipment to be used for other, more rapid experiments. The slow degradation rate at low pH values meant that some experiments could not be continued beyond about 80% residual drug concentration. The extent of degradation and duration of the experiment are therefore included in Table 24 together with the apparent first order rate constants. The whole profile is shown graphically in Figure 40.

The drug is evidently most stable in the region pH 3 - 4 whilst the degradation rate reaches a peak at pH 9. TLC examination of solutions degraded at each pH revealed a similar pattern of breakdown under all conditions as shown in Figure 41.

| pH   | $k(s^{-1})$                                     | Std.Dev.                                       | Duration of experiment | Final residual drug concentration |
|------|---|--|------------------------|-----------------------------------|
| 0.85 | $7.28 \times 10^{-7}$                           | $3.14 \times 10^{-8}$                          | 260 hours              | 38%                               |
| 1.5  | $4.86 \times 10^{-8}$                           | $2.26 \times 10^{-9}$                          | 138 days               | 55%                               |
| 2.0  | $3.22 \times 10^{-8}$                           | $2.94 \times 10^{-9}$                          | 130 days               | 80%                               |
| 3.0  | $8.08 \times 10^{-9}$                           | $1.33 \times 10^{-9}$                          | 294 days               | 80%                               |
| 5.0  | $5.83 \times 10^{-8}$                           | $4.58 \times 10^{-9}$                          | 450 hours              | 90%                               |
| 6.4  | $5.03 \times 10^{-7}$                           | $2.19 \times 10^{-8}$                          | 400 hours              | 50%                               |
| 7.0  | $9.94 \times 10^{-7}$<br>$10.75 \times 10^{-7}$ | $3.50 \times 10^{-8}$<br>$3.92 \times 10^{-8}$ | 200 hours              | 40%                               |
| 7.5  | $1.75 \times 10^{-6}$                           | $2.74 \times 10^{-8}$                          | 140 hours              | 40%                               |
| 8.0  | $3.64 \times 10^{-6}$                           | $6.61 \times 10^{-8}$                          | 65 hours               | 43%                               |
| 8.5  | $5.22 \times 10^{-6}$                           | $1.02 \times 10^{-7}$                          | 67 hours               | 28%                               |
| 9.0  | $7.00 \times 10^{-6}$<br>$6.81 \times 10^{-6}$  | $7.75 \times 10^{-8}$<br>$6.22 \times 10^{-8}$ | 65 hours               | 27%                               |
| 10.0 | $5.06 \times 10^{-6}$                           | $1.80 \times 10^{-7}$                          | 75 hours               | 29%                               |

Table 24. The effect of pH on the apparent first order rate constant for the degradation of 0.5% salbutamol sulphate solution at 70°.

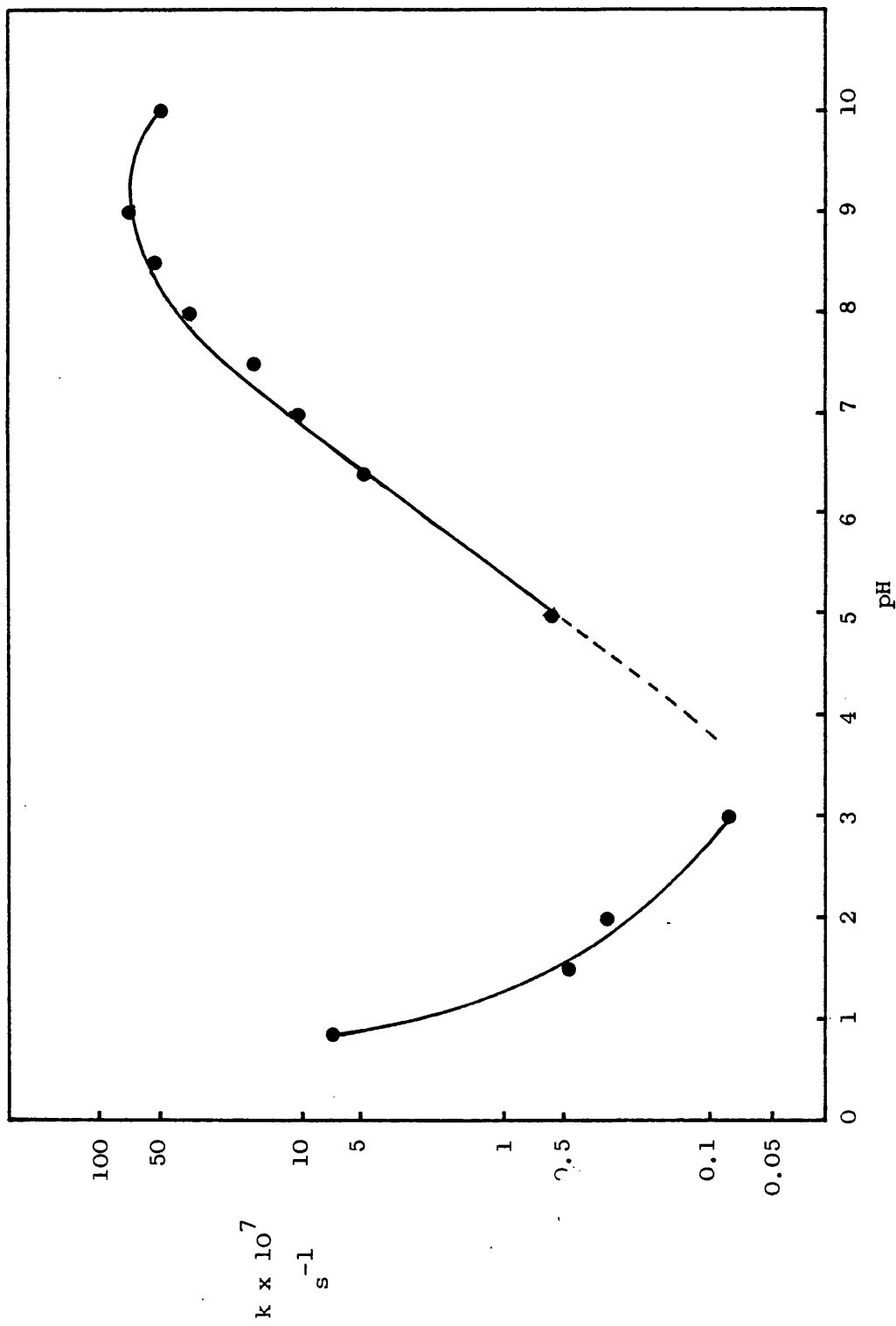


Fig. 40. pH-stability profile of 0.5% salbutamol sulphate solution at 70°C

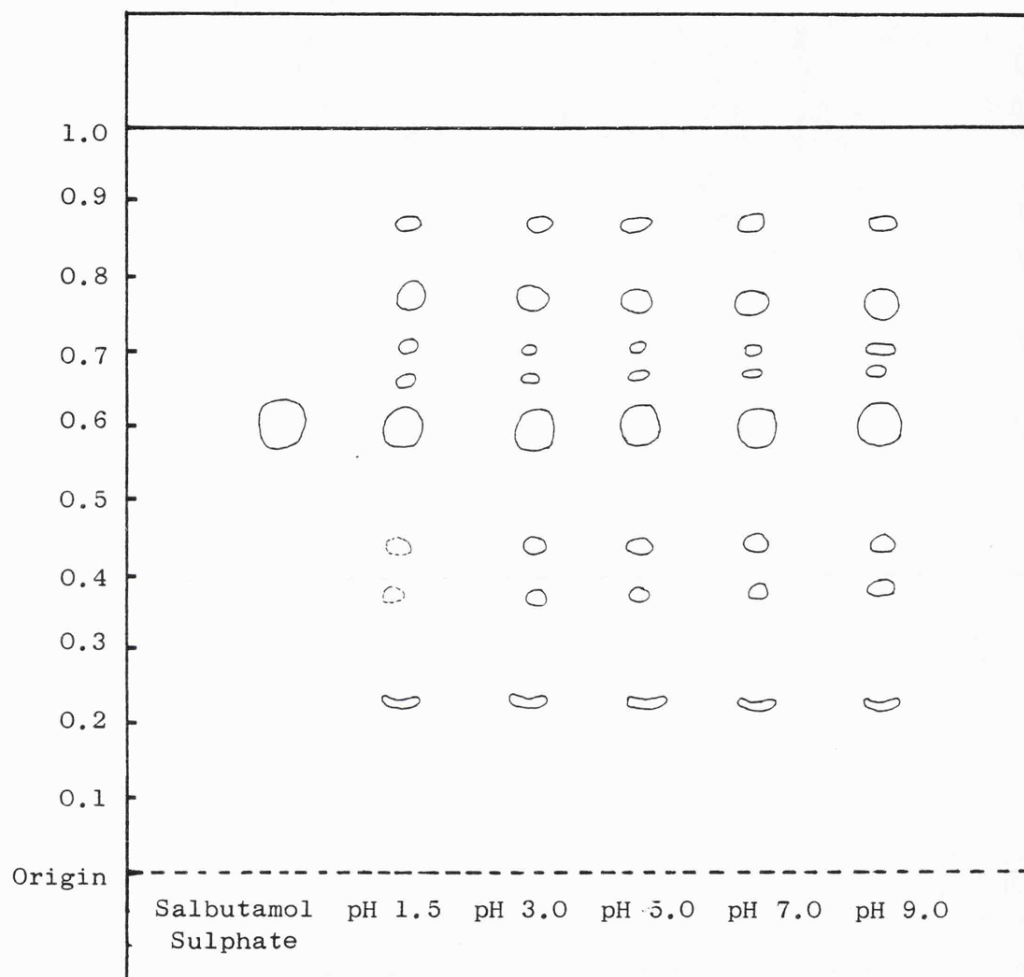


Fig. 41. Diagrammatic TLC plate of salbutamol sulphate solutions degraded at various pH values.

f) The effect of buffer salts

Three commonly used buffers were examined at a concentration of 0.1M, namely sodium acetate, sodium dihydrogen phosphate and citric acid. The solutions were maintained at pH 7.0 by pH-statting. A concentration of 0.5% salbutamol sulphate was used at a temperature of 70°. The results are depicted in Figure 42 and the apparent first order rate constants are presented in Table 25.

| Buffer salt | $k(s^{-1}) \times 10^6$ | Std. Dev. $\times 10^8$ |
|-------------|-------------------------|-------------------------|
| None        | 0.994                   | 3.50                    |
|             | 1.08                    | 3.92                    |
| Phosphate   | 1.49                    | 6.78                    |
| Acetate     | 0.767                   | 1.48                    |
| Citrate     | 1.03                    | 3.67                    |
|             | 1.11                    | 7.67                    |

Table 25. The effect of 0.1M buffers on the apparent first order rate constant for the degradation of 0.5% salbutamol sulphate solutions at pH 7 and 70°.

The lines obtained from the experiments using 0.1M citrate were not linear for either replicate, as can be seen from Figure 43. It appears that the degradation slows down when the drug concentration falls to about 70% of the initial value and then accelerates again below 60% residual salbutamol sulphate. The initial and final rates appear to be approximately equal and so, in addition to the overall

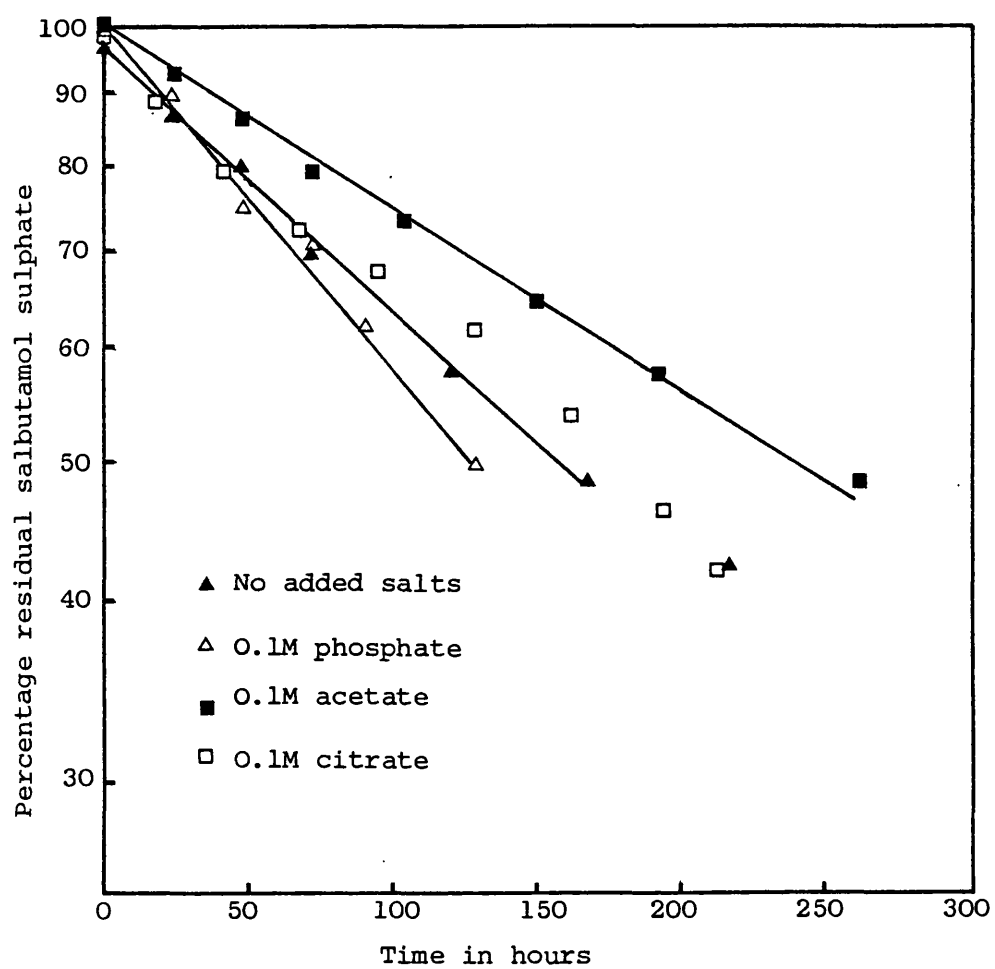


Fig. 42. The effect of buffer salts on the degradation of 0.5% salbutamol sulphate at pH 7.0 and 70°.



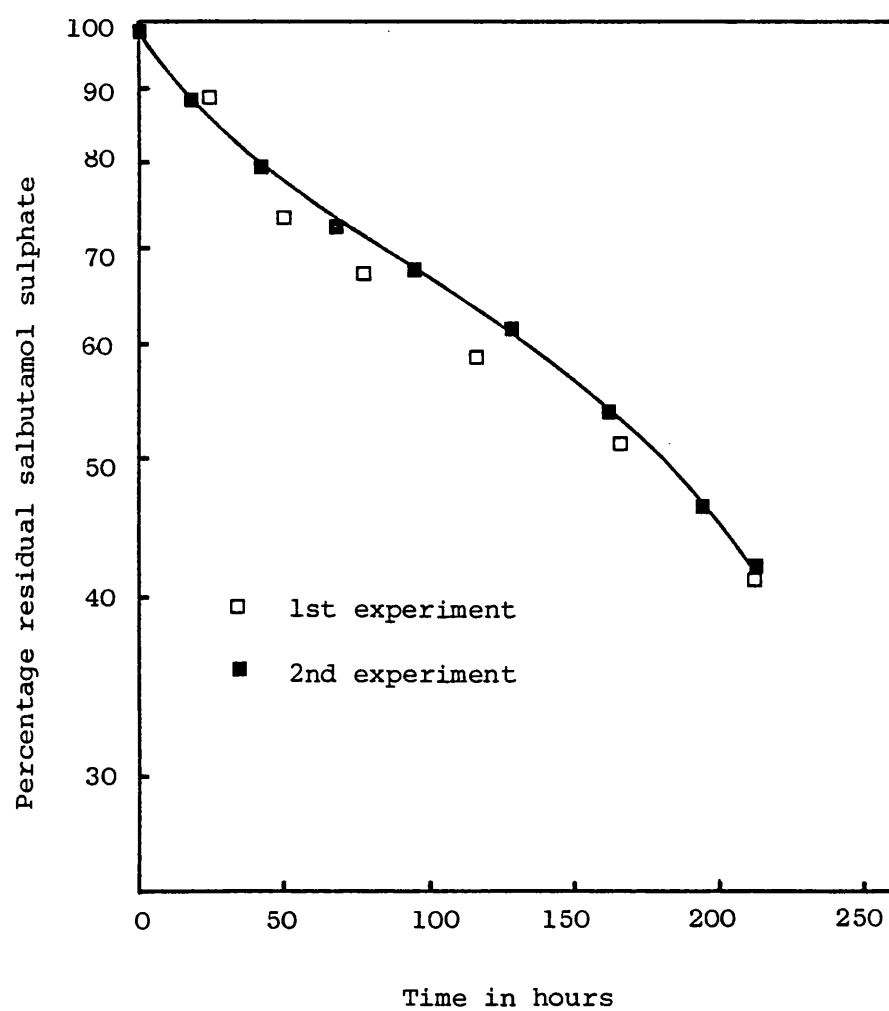


Fig. 43. The effect of 0.1M citrate on the degradation of 0.5% salbutamol sulphate solution at pH 7.0 and 70°.

rate constant from the total data (Table 25), the individual constants from the first four and last four data points of one line were also calculated. The values obtained were  $1.24 \times 10^{-6} \pm 7.86 \times 10^{-8} \text{ s}^{-1}$  and  $1.26 \times 10^{-6} \pm 4.83 \times 10^{-8} \text{ s}^{-1}$ . These were subjected to a Student's t-test which gave a t-value of 0.24, the tabulated value at the 5% probability level being 2.776. The initial and final rate constants are therefore not significantly different but are about 20% greater than the overall rate which is reduced by the intermediate lag phase.

The phosphate results show a similar, but less marked discontinuity, the overall effect being an acceleration of the rate of salbutamol breakdown, whilst the graph for the experiment containing 0.1M acetate is linear and shows a clear stabilising effect.

g) The effect of EDTA

Oxidation reactions are notoriously sensitive to catalysis by trace metal ions, particularly copper, which are often present as contaminants, even in high grade materials. These ions are chelated by ethylene diamine tetra-acetic acid (EDTA) and are thereby prevented from exerting a catalytic effect.

0.05% EDTA was used with a salbutamol sulphate concentration of 0.5% at pH 7.0 and 70°, and the results are summarised in Table 26.

| WITH EDTA                    |                            |                        | WITHOUT EDTA                 |                            |                        |
|------------------------------|----------------------------|------------------------|------------------------------|----------------------------|------------------------|
| $k(s^{-1})$<br>$\times 10^6$ | Std. Dev.<br>$\times 10^8$ | No. of<br>points,<br>n | $k(s^{-1})$<br>$\times 10^6$ | Std. Dev.<br>$\times 10^8$ | No. of<br>points,<br>n |
| 1.43                         | 11.8                       | 5                      | 0.994                        | 3.50                       | 6                      |
| 1.51                         | 5.47                       | 7                      | 1.08                         | 3.92                       | 7                      |

Table 26. The effect of 0.05% EDTA on the apparent first order rate constant for the degradation of 0.5% salbutamol sulphate solutions at pH 7.0 and 70°.

A Student t-test on each pair of results gives a value of 1.513 (without EDTA) and 0.652 (with EDTA). These are both smaller than the tabulated values of t at the 5% significance level and so the data are reproducible under each set of conditions, the replicates not being significantly different. However, there is obviously a difference between the values obtained with EDTA and those without. Surprisingly, the EDTA accelerates the breakdown of the drug instead of stabilising it as expected. This implies that either a stabilising ion is being chelated, or that the EDTA is exerting a direct catalytic action of its own.

#### h) The effect of sugars

The sugars studied were sucrose, which is the principal constituent of the oral salbutamol syrup, glucose and fructose.

Experiments were carried out using a drug concentration of 0.5% at 70° at both pH 7.0 and 3.5. The first experiments used a sugar concentration of 10% at pH 7.0 because the

degradation rate of simple salbutamol solutions is quite rapid at this pH. Later work examined the effect of 50% sugar at pH 3.5, conditions which approximate to those in the commercial syrup.

Figure 44 illustrates the results obtained at pH 7 using 10% sucrose and 10% glucose. The glucose results give a distinctly curved line and so the rate constant in Table 27 was calculated from the first five data points only. All the data were used in the calculation of the apparent first order rate constants of the other two lines. The graph and table clearly demonstrate that glucose increased the rate of salbutamol breakdown by a factor of two but sucrose had no effect.

The results obtained at pH 3.5 using 50% sugar are depicted in Figure 45. Since the lines deviate from linearity, the number of points,  $n$ , used in the calculation of the apparent first order rate constants is included in Table 28, which presents the calculated values. The effect of sugar concentration was also examined at pH 3.5 using sucrose. The rate constants are included in Table 28 and the results are shown graphically in Figure 46.

The most striking difference between the results at pH 7.0 and 3.5 is in the effect of sucrose. Although this sugar had no effect on the rate of salbutamol breakdown at the higher pH, at 3.5 the rate was increased substantially. The two monosaccharides, glucose and fructose also increased

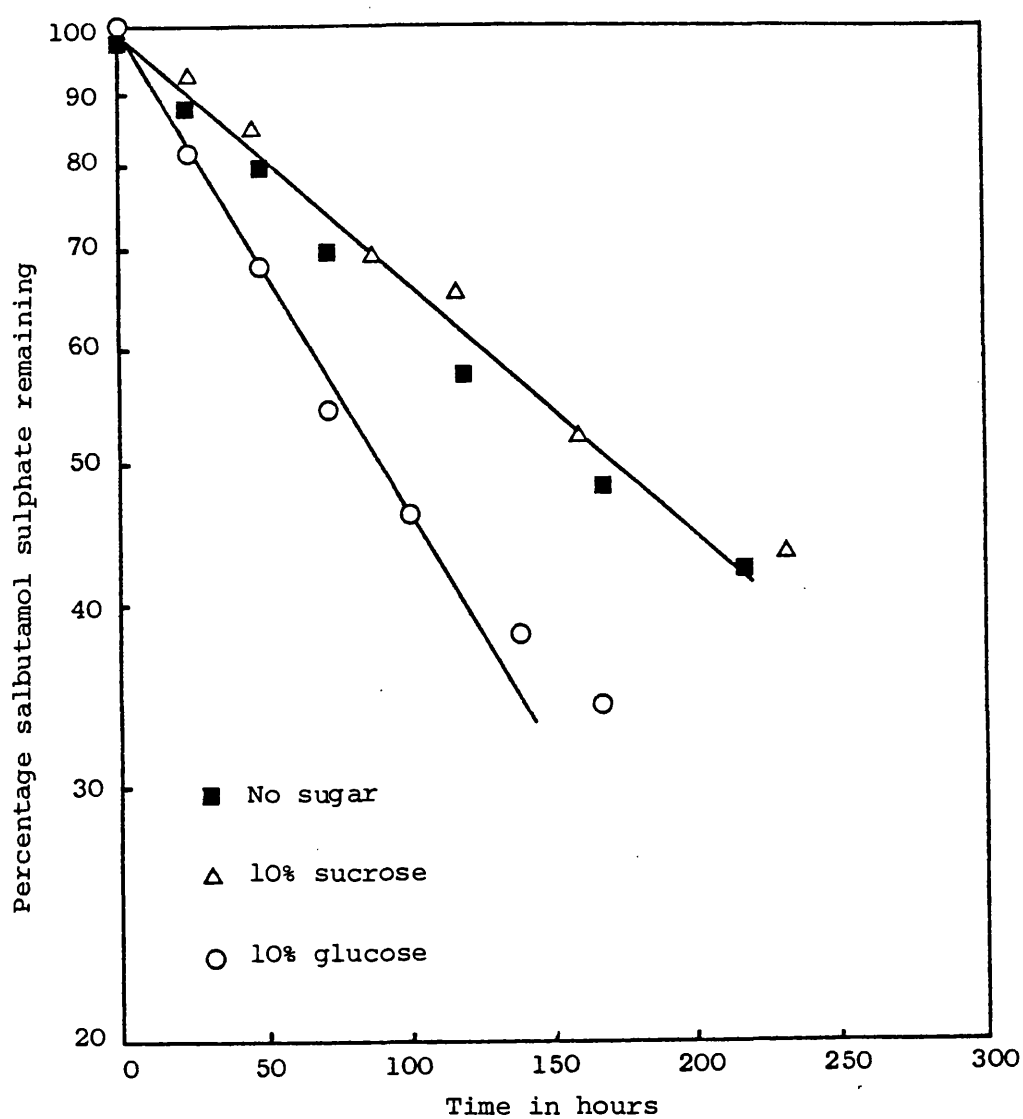


Fig. 44. Degradation of 0.5% salbutamol sulphate solution at pH 7 and 70° in the presence of 10% sugar

| Sugar   | Apparent first order<br>rate constant ( $s^{-1}$ ) $\times 10^6$ | Std.Dev. $\times 10^8$ |
|---------|--|------------------------|
| None    | 0.994  | 3.50                   |
|         | 1.08   | 3.92                   |
| Sucrose | 1.02   | 4.72                   |
| Glucose | 2.19   | 8.44                   |

Table 27. Apparent first order rate constants for the degradation of 0.5% salbutamol sulphate solutions at pH 7.0 and 70° in the presence of 10% sugar.

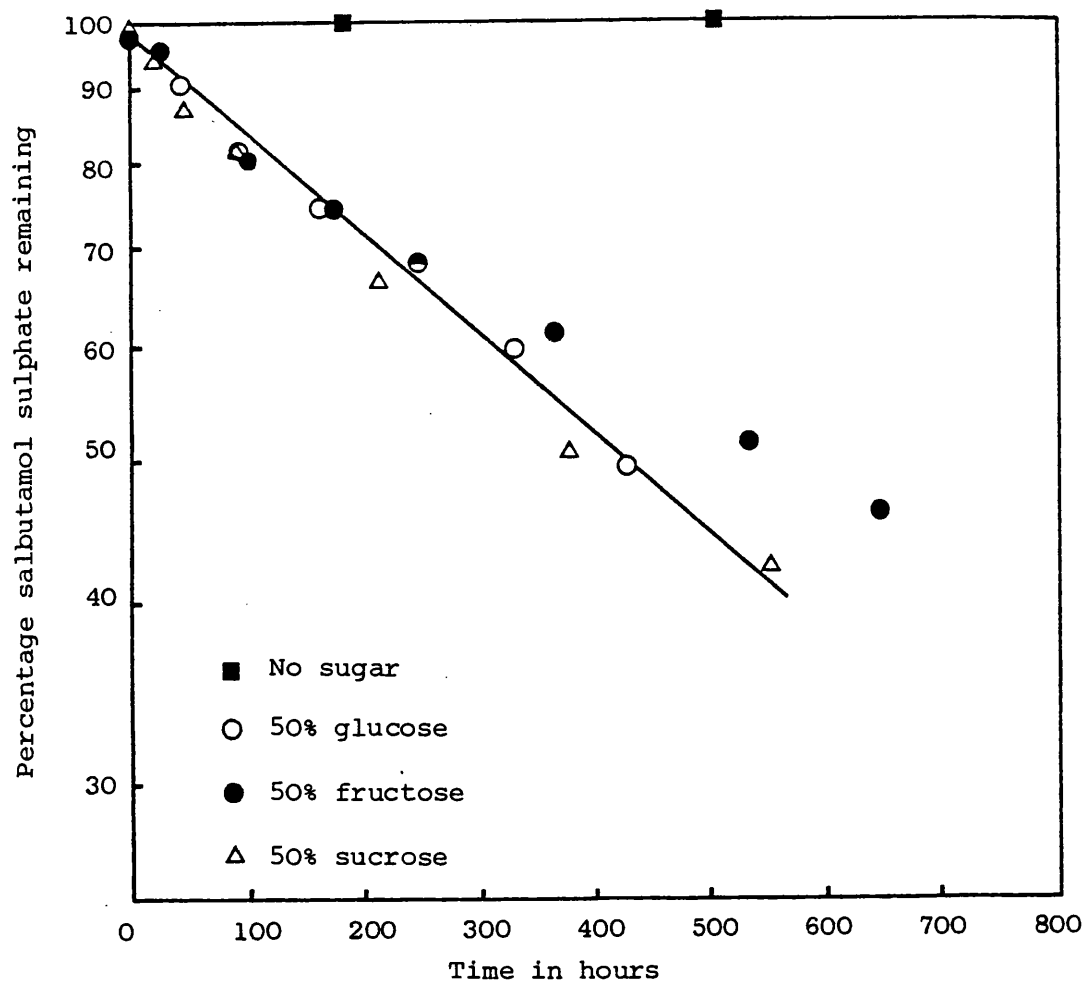


Fig. 45. Degradation of 0.5% salbutamol sulphate solution at pH 3.5 and 70° in the presence of 50% sugar

| Sugar concentration<br>% w/v | pH control | $k(s^{-1}) \times 10^7$ | Std.Dev. | n |
|------------------------------|------------|-------------------------|----------|---|
| 50% glucose                  | pH stat    | 3.97                    | 2.00     | 6 |
| 50% glucose                  | citrate    | 4.14                    | 1.01     | 3 |
| 50% fructose                 | pH stat    | 4.22                    | 4.19     | 5 |
| 50% sucrose                  | pH stat    | 4.81                    | 1.75     | 6 |
| 50% sucrose                  | citrate    | 4.33                    | 1.93     | 3 |
| 10% sucrose                  | pH stat    | 0.544                   | 0.308    | 6 |

Table 28. Apparent first order rate constants for the degradation of 0.5% salbutamol sulphate solutions at pH 3.5 and 70° in the presence of sugars.

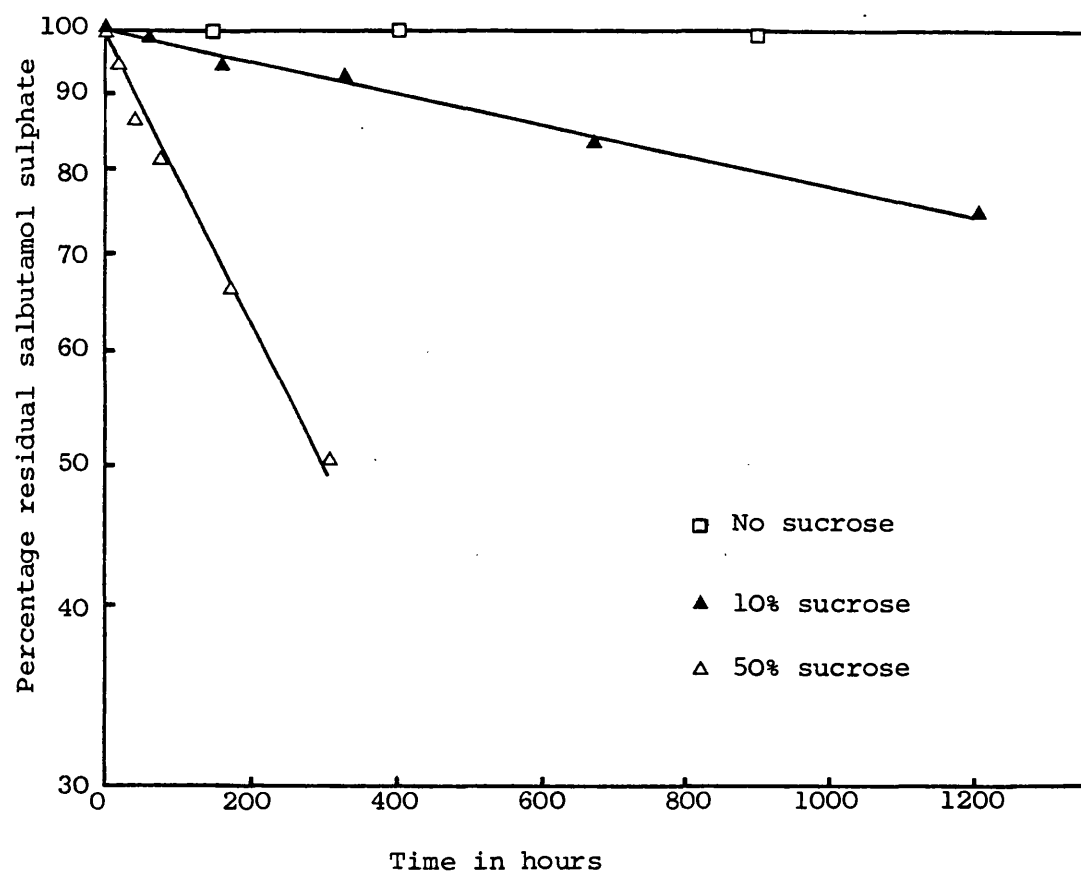


Fig. 46. The effect of sucrose concentration on the degradation of 0.5% salbutamol sulphate solution at pH 3.5 and 70<sup>o</sup>,

the degradation to approximately the same extent as sucrose, resulting in a half-life for the drug of about 400 hours. In the absence of sugar no detectable breakdown occurs over a similar time period, as illustrated in Figure 45. Figure 46 shows that the accelerating effect of sucrose at this pH is concentration dependent and the rate constant data in Table 28 reveal a eight-fold increase in the rate constant for a five-fold increase in sucrose concentration.

The HPLC traces of the degraded samples containing glucose and fructose showed the presence of an additional peak ( $k' = 0.40$ ) which eluted before salbutamol (Fig. 47). A similar peak was observed in pH 3.5 samples containing sucrose but not in sucrose samples at pH 7.0. Since no acceleration of the degradation rate was observed in the presence of sucrose at pH 7.0, the new peak must represent a new decomposition product, the formation of which increases the rate of loss of salbutamol sulphate, and is probably the result of a reaction between the drug and excipient.

i) The effect of citrate buffer in the presence of sugars

The commercial syrup contains a citrate buffer to control the pH and so, although citrate had been shown to have little effect at pH 7 (Section 2.12f), its effect at pH 3.5 in the presence of 50% glucose and 50% sucrose was examined. The buffer was the same as that used in the formulated product and consisted of 1.0% citric acid and 0.575% sodium citrate. The apparent first order rate constants calculated from the results, which are plotted in Figure 48,



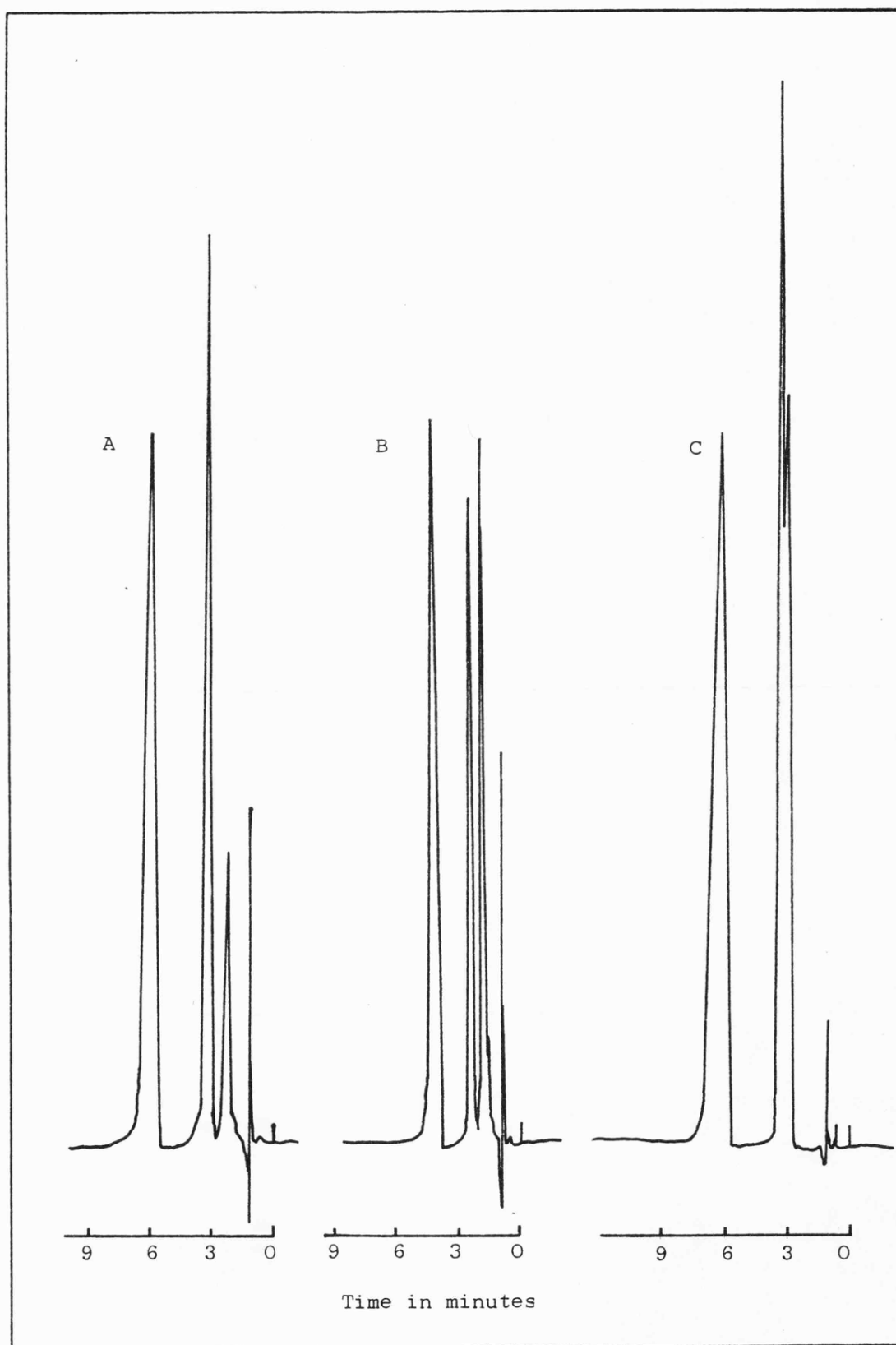


Fig. 47. HPLC traces of degraded 0.5% salbutamol sulphate solutions containing 50% polyhydric excipients at pH 3.5 and 70°.

Conditions as Fig. 21C.

- A. Glucose (200 hours)
- B. Sorbitol (350 hours)
- C. Glycerol (160 hours)

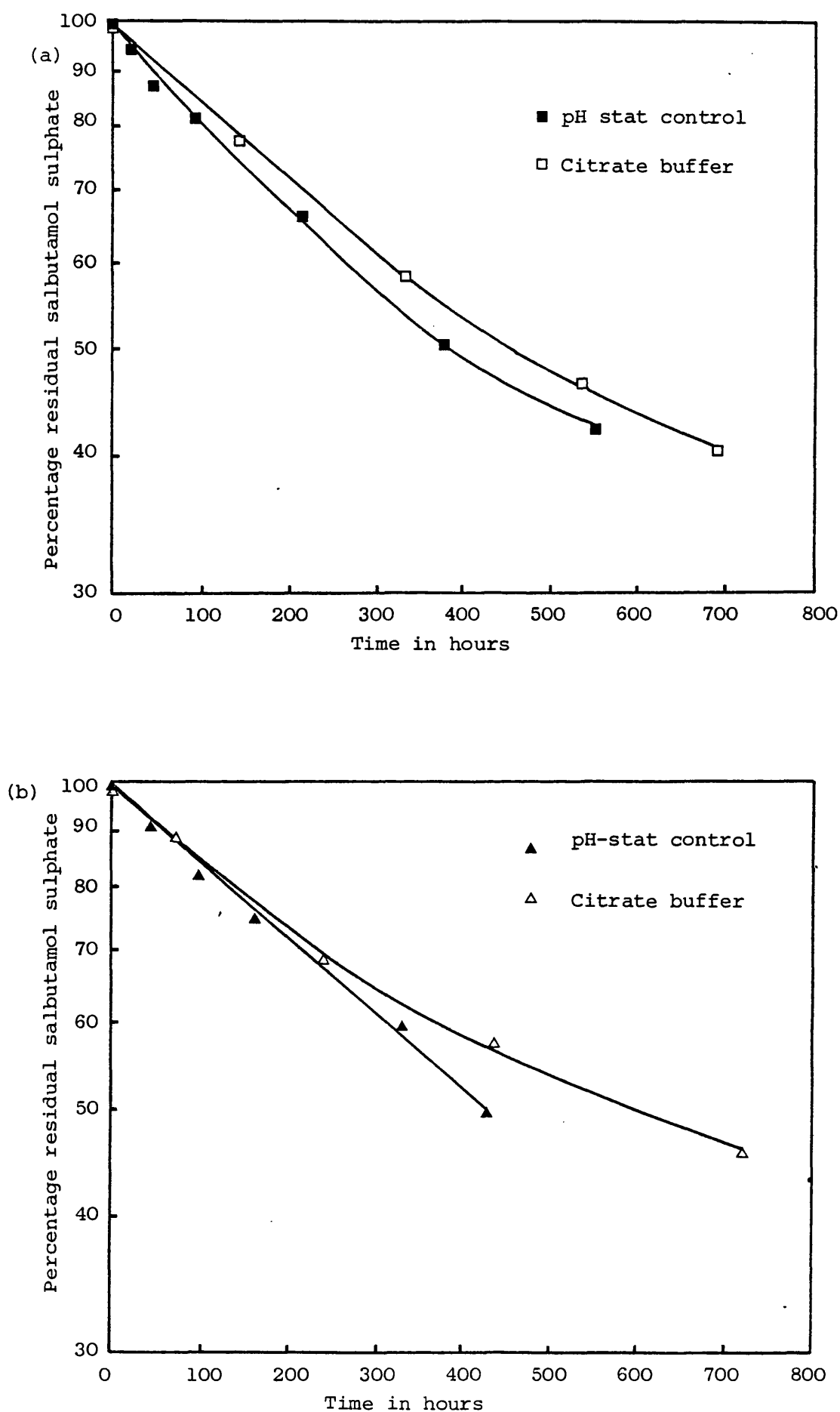
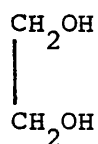


Fig. 48. The effect of citrate buffer on the degradation of 0.5% salbutamol sulphate in the presence of 50% sugar at pH 3.5 and 70° (a) sucrose (b) glucose

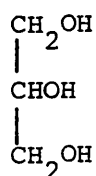
are also included in Table 28. As before, the number of points used in the computation of each line is tabulated. A Bartlett test (Appendix 1) on the data for 50% sugar at pH 3.5 gave a  $\chi^2$  value of 6.31. The tabulated value at the 5% probability level for 4 degrees of freedom is 9.488 and so the initial rate constants do not differ significantly. However, it is apparent from the graphs (Figs. 45 and 48) that differences between the sugars do exist and that citrate has a small stabilising effect which only becomes apparent when the residual drug concentration has fallen to about 70%.

j) The effect of polyhydric excipients

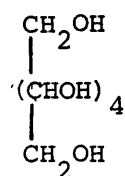
In order to determine the nature of the reaction between sugars and salbutamol, several types of compound with different functional groups were studied. One group of compounds consisted of propylene glycol, glycerol and sorbitol. These all have a single type of functional group, the alcoholic hydroxyl, but differ in the number of such groups per molecule.



Propylene glycol



Glycerol



Sorbitol

Initially, propylene glycol and glycerol were used at a concentration of 10% with a drug concentration of 0.5% at pH 7.0 and 70°. Figure 49 illustrates the results obtained. The initial rapid loss in the presence of propylene glycol

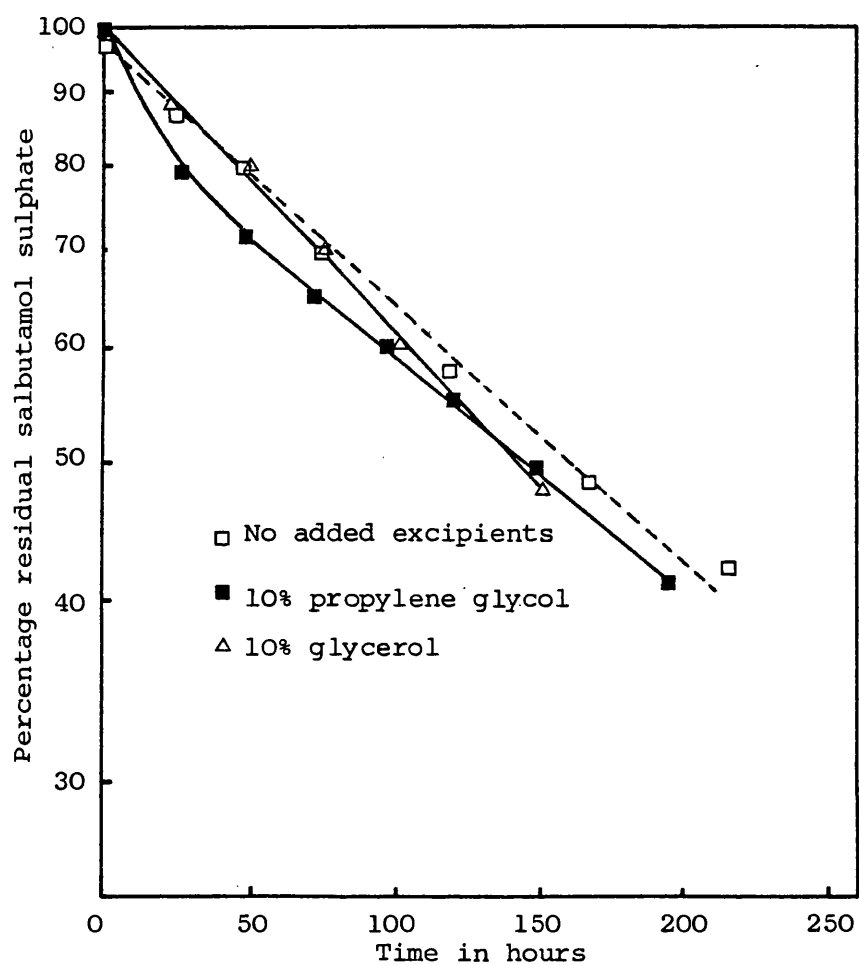


Fig. 49. The effect of polyhydric alcohols on the degradation of 0.5% salbutamol sulphate at pH 7.0 and 70°.

was estimated to have a rate constant of  $2.4 \times 10^{-6} \text{ s}^{-1}$ .

This then falls to a value of  $1.03 \times 10^{-6} \text{ s}^{-1} \pm 1.66 \times 10^{-8}$ .

The more linear glycerol graph gave a rate constant of  $1.33 \times 10^{-6} \text{ s}^{-1} \pm 7.83 \times 10^{-8}$ , an increase of approximately 30% over that for a simple solution of pH 7.

Glycerol and sorbitol were then examined at the 50% level at pH 3.5, as a comparison with the sugar effects under these conditions. The results are depicted in Figure 50 from which the initial apparent first order rate constants were calculated. Like the sugars, these polyhydric alcohols accelerated the breakdown of salbutamol sulphate and an additional peak appeared in the analytical chromatogram. The peak from the sorbitol samples had a  $k'$  value of approximately 1.0 whilst the glycerol product could only just be separated from salbutamol, having  $k' = 1.75$  (Figure 47).

| Excipient | $k(\text{s}^{-1})$<br>$\times 10^7$ | Std. Dev.<br>$\times 10^8$ | n |
|-----------|-------------------------------------|----------------------------|---|
| Glycerol  | 4.03                                | 2.24                       | 4 |
| Sorbitol  | 5.25                                | 2.63                       | 5 |

Table 29. The apparent first order rate constants for the degradation of 0.5% salbutamol sulphate solution at pH 3.5 and  $70^\circ$  in the presence of 50% polyhydric alcohols.

A Bartlett test was used to compare the sorbitol rate constant with those obtained from the experiments using 50%

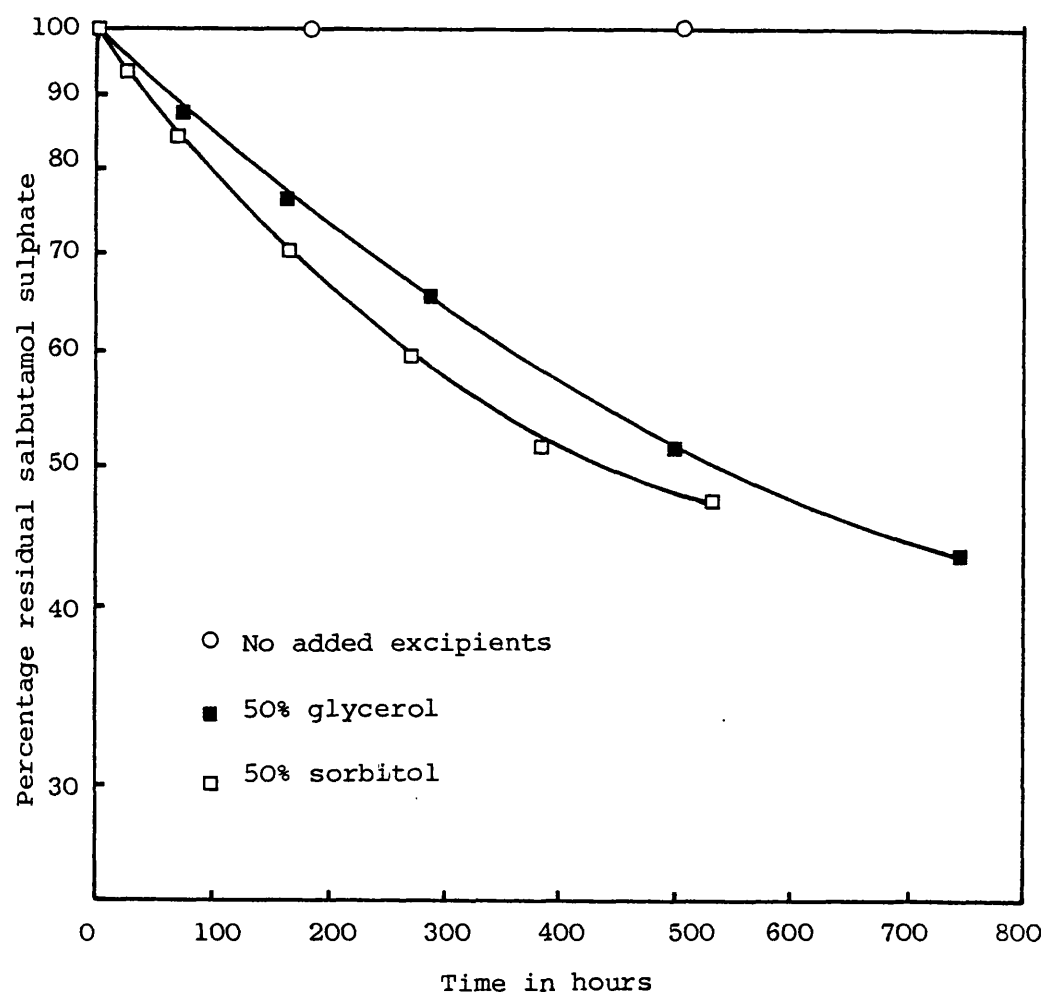


Fig. 50 . The effect of polyhydric alcohols on the degradation of 0.5% salbutamol sulphate at pH 3.5 and 70°.

sugars. The  $\chi^2$  value obtained was 18.09 which is greater than the tabulated value of 11.07. This confirms that the degradation rate in the presence of 50% sorbitol at pH 3.5 is significantly greater than in the presence of sugars under similar conditions.

k) The effects of Glyceraldehyde and glyceric acid

The most reactive centre in a sugar molecule is the anomeric carbonyl carbon atom (Section 1.6) and so this is a likely point of interaction with salbutamol. The carbonyl group is also sensitive to oxidation, being converted to a carboxylic acid. If this were to occur under the experimental conditions used in this study, the reactive species could be the aldonic acid instead of the aldose.

Although not usually classified as a sugar, glyceraldehyde bears the same relationship to glucose as glycerol does to sorbitol and so may be considered to be the simplest aldose. It differs from most sugars in not being able to form a cyclic structure due to the brevity of the carbon chain. Oxidation of the carbonyl group produces glyceric acid and so the stability of salbutamol sulphate in the presence of these two compounds was examined, in an attempt to determine the nature of the active functional group in salbutamol - sugar reactions.



Glyceraldehyde



Glyceric acid

Solutions containing 0.5% salbutamol sulphate and 5% of the test compound were used. The low solubility of glyceraldehyde precluded the use of concentrations as high as 10%, as had been used for glycerol and the sugars. The pH was 3.5 and the temperature  $70^{\circ}$ , as before.

Figure 51 demonstrates the unexpected nature of the results. Glyceraldehyde, whilst slow to produce an effect at first, rapidly accelerated the breakdown of the drug whereas glyceric acid, after a similar time interval of about 50 hours, produced a plateau with about 73% residual salbutamol sulphate.

1) The effect of 5-hydroxymethylfurfural (5-HMF)

5-HMF is a major product of the breakdown of sugars and is believed to be an intermediate in many sugar reactions (58, 59, 106-108). Three concentrations were tested, 0.015%, 0.03%, and 0.10%, all at pH 7 and  $70^{\circ}$  with a salbutamol sulphate concentration of 0.5%. The concentrations were chosen to cover the likely range of 5-HMF production from 10% glucose at pH 7 (58, 107, 108). Formation of 5-HMF at pH 3.5 will be minimal since this is the region of maximum glucose stability (56-58).

The results indicate (Fig. 52) that 5-HMF does not have a marked effect on the degradation of salbutamol sulphate but at low levels may have a stabilising effect. The graph for 0.015% 5-HMF is distinctly curved and so an apparent first order rate constant was calculated using the first six



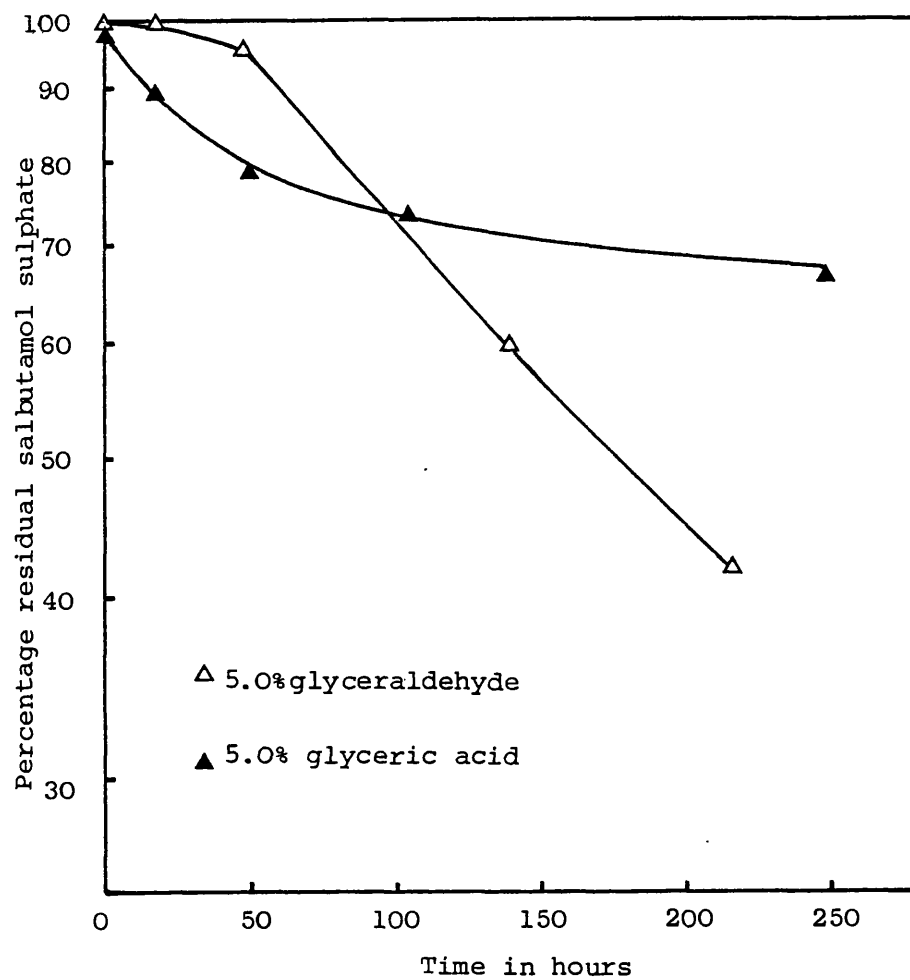


Fig. 51. The effect of glycerinaldehyde and glyceric acid on the degradation of 0.5% salbutamol sulphate solution at pH 3.5 and 70°,

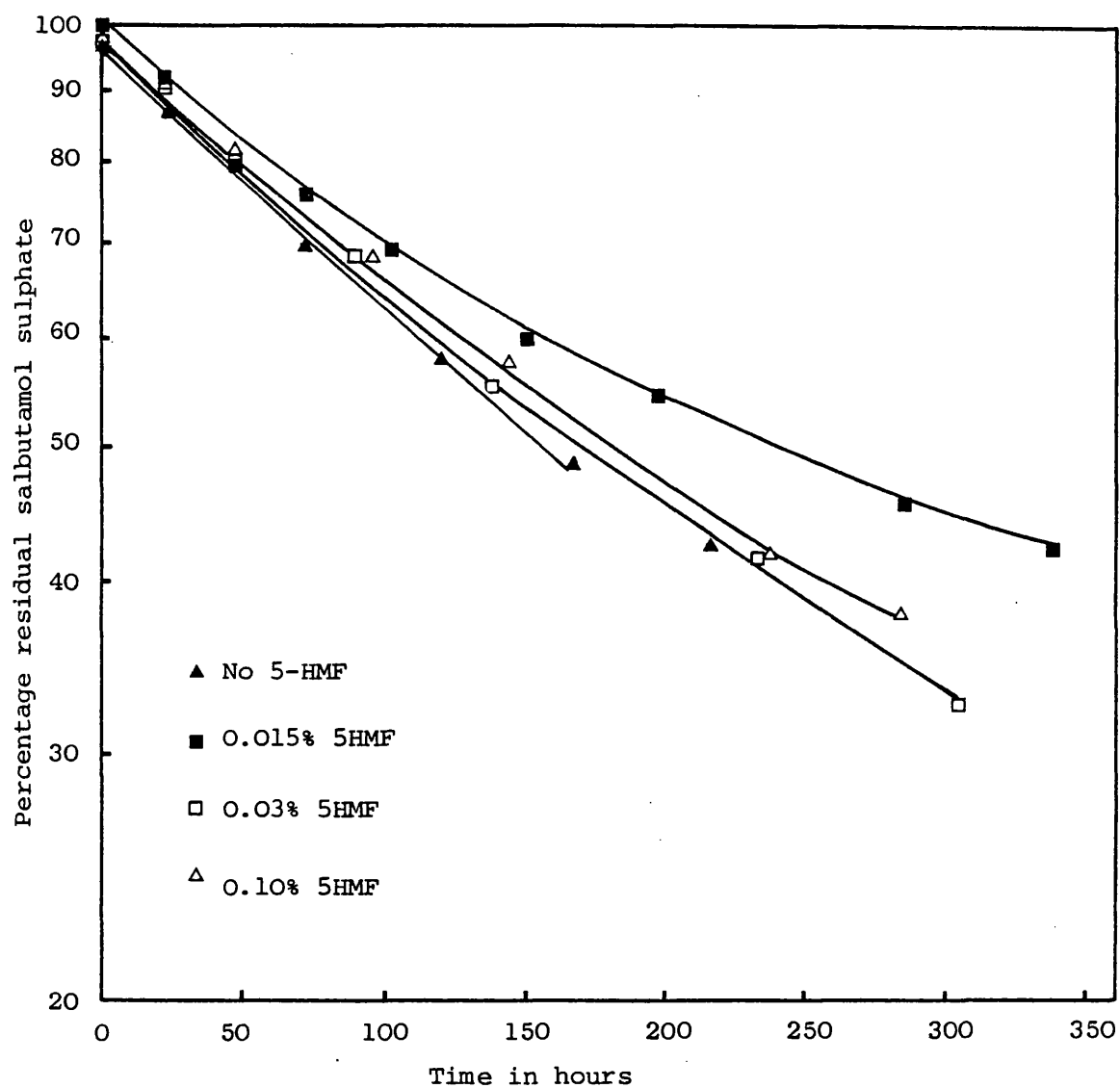


Fig. 52. The effect of 5-hydroxymethylfurfural (5-HMF) on the degradation of 0.5% salbutamol sulphate solution at pH 7.0 and 70°.

data points only. All the points were used for the other concentrations and the results are summarised in Table 30.

| Concentration of<br>5-HMF (% w/v) | $k(s^{-1})$<br>$\times 10^7$ | Std. Dev.<br>$\times 10^8$ |
|-----------------------------------|------------------------------|----------------------------|
| 0                                 | 9.94                         | 3.50                       |
|                                   | 10.75                        | 3.92                       |
| 0.015                             | 9.39                         | 6.47                       |
| 0.03                              | 10.06                        | 3.58                       |
| 0.10                              | 10.00                        | 2.09                       |

Table 30. The effect of 5-HMF on the apparent first order rate constant for the degradation of 0.5% salbutamol sulphate at pH 7 and 70°.

The three rate constants were compared with each other using Bartlett's test (Appendix 1) which gave a value of 1.416 for  $\chi^2$ . The tabulated value at the 5% probability level is 5.991 and so the three constants do not differ significantly. The data were also compared with that obtained for 0.5% salbutamol sulphate solutions at pH7 and 70° in the absence of 5-HMF. The calculated  $\chi^2$  value in this test was 5.475 and the corresponding tabulated value is 9.488. The data therefore do not show statistically significant variation at the 5% probability level.

## Section 3.

IDENTIFICATION OF BREAKDOWN PRODUCTS3.1 Introduction

Attempts were made to isolate and identify the major degradation products of salbutamol sulphate in aqueous solution, using the method described in Section 2.7. However, in simple solutions, from the large number of compounds formed (Fig. 4.1), only one could be isolated in sufficient quantity for further study. This formed the largest spot on TLC, apart from salbutamol itself, and had an  $R_f$  value of 0.75 - 0.80.

The compound was isolated as a pale yellow or colourless solid. Its solution (methanolic or aqueous) darkened on standing, turning deep yellow and then brown. It was examined by both  $^1\text{H}$ - and  $^{13}\text{C}$ -nmr and also subjected to mass spectrometry, in order to determine its structure.

Products which were formed, as a result of the interaction between salbutamol sulphate and sugars underwent breakdown during attempted purification. The only compound which was isolated exhibited the characteristics of salbutamol and not the original interaction product when examined by  $^1\text{H}$ -nmr and TLC. However, the product from the interaction of salbutamol sulphate and glycerol was considerably more stable and was isolated and examined by  $^1\text{H}$ - and  $^{13}\text{C}$ -nmr.

The nmr spectra of salbutamol were determined initially

for reference purposes. The  $^1\text{H}$ -nmr spectrum of salbutamol is depicted in Fig. 53. The large peak at  $\delta 1.2$  is due to the nine t-butyl protons and was used as the basis for determination of the peak area ratios. The data are presented in Table 31, together with the peak assignments.

| $\delta$ (ppm) | Peak Splitting | Coupling constant<br>J (Hz) | Assignment                     |
|----------------|----------------|-----------------------------|--------------------------------|
| 1.2            | singlet        | -                           | $-\text{C}(\text{CH}_3)_3$ (g) |
| 2.8            | doublet        | 6.0                         | $-\text{CH}_2\text{N}-$ (f)    |
| 4.45           | singlet        | -                           | $-\text{CH}_2\text{OH}$ (e)    |
| 4.8            | triplet        | 6.0                         | $-\text{CH}(\text{OH})-$ (d)   |
| 6.75           | doublet        | 7.8                         | Aromatic (c)                   |
| 7.05           | double doublet | 7.0, 3.0                    | Aromatic (b)                   |
| 7.3            | doublet        | 2.0                         | Aromatic (a)                   |

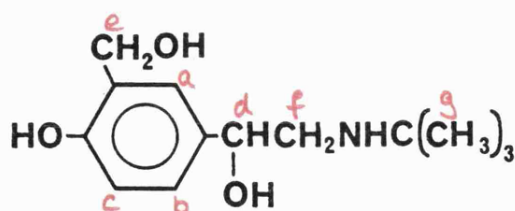
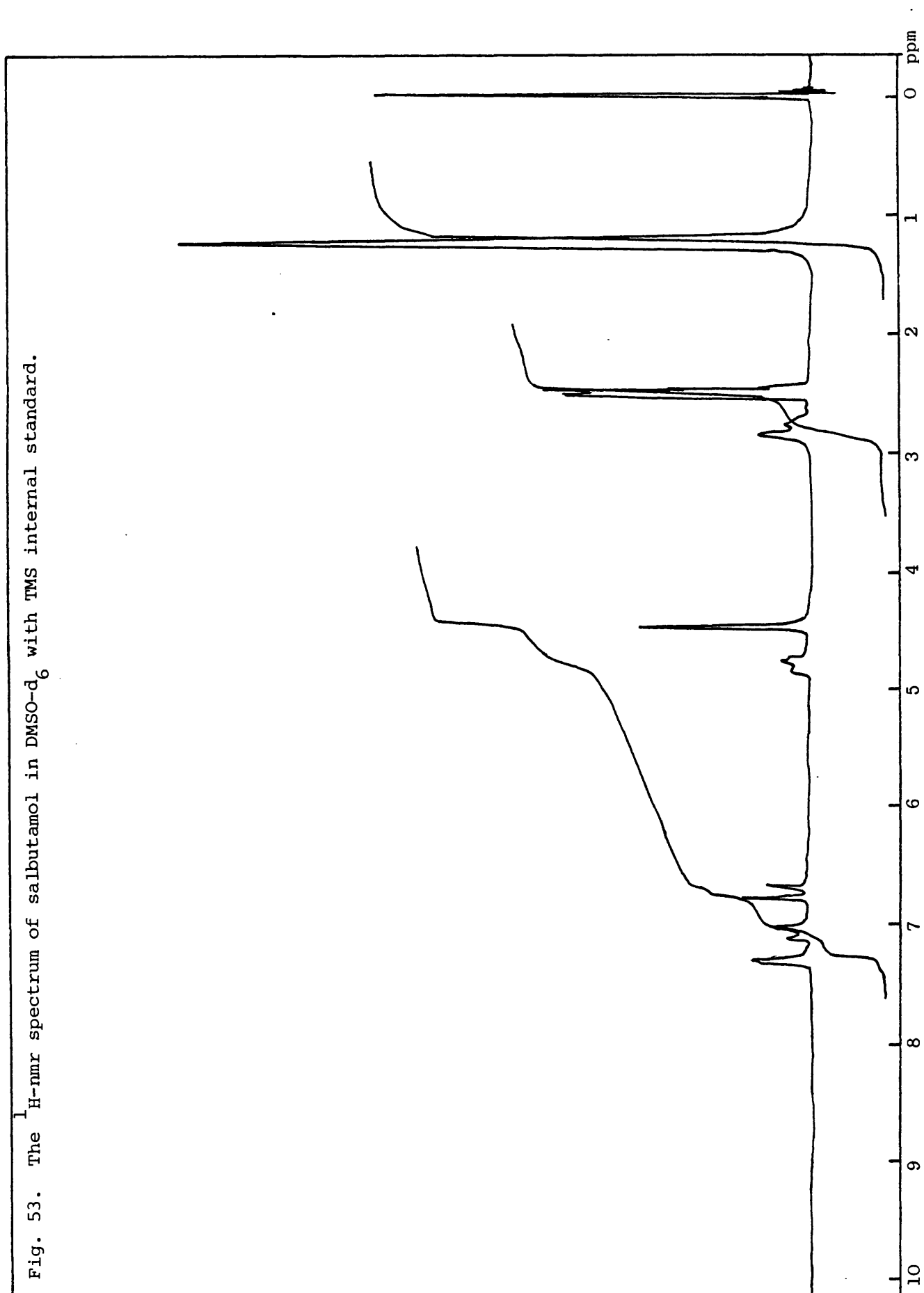


Table 31.  $^1\text{H}$ -nmr data for salbutamol in  $\text{DMSO}-d_6$  with TMS internal standard.

Noise decoupled and the off-resonance  $^{13}\text{C}$ -nmr spectra gave signals which were unequivocally assigned (Table 32), the assignments being in accord with the data published recently by Pasaribu and Brophy (130).



| Chemical shift | C-H<br>Coupling | Assignment                             |
|----------------|-----------------|--|
| 153.3          | singlet         | Aromatic ( <b>d</b> )                  |
| 134.4          | singlet         | Aromatic ( <b>a</b> )                  |
| 127.8          | singlet         | Aromatic ( <b>e</b> )                  |
| 125.1          | doublet         | Aromatic } ( <b>b</b> ) + ( <b>f</b> ) |
| 124.9          | doublet         |  |
| 114.1          | doublet         | Aromatic ( <b>c</b> )                  |
| 72.3           | doublet         | -CH(OH)-                               |
| 58.6           | triplet         | -CH <sub>2</sub> OH                    |
| 50.5           | triplet         | -CH <sub>2</sub> NH-                   |
| 49.5           | singlet         | -NHC(Me) <sub>3</sub>                  |
| 28.7           | quadruplet      | -C(CH <sub>3</sub> ) <sub>3</sub>      |

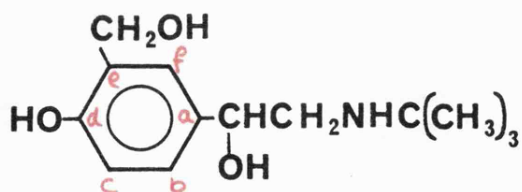
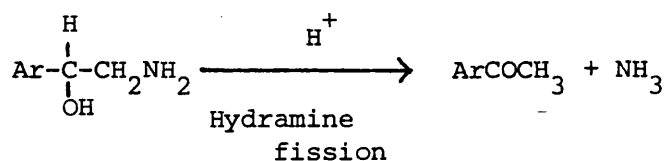


Table 32. <sup>13</sup>C-nmr data for salbutamol in DMSO-d<sub>6</sub> with TMS as internal standard

### 3.2 The major degradation product in simple solution

The  $^1\text{H}$ -nmr spectrum of the principal degradation product of salbutamol sulphate in aqueous solution is depicted in Fig. 54. The main difference between this spectrum and that of salbutamol (Fig. 53) is the additional peak at  $\delta 3.8$ . The integration suggests the presence of 5-6 aromatic protons, that is, two tri-substituted aromatic nuclei, but only one t-butyl group. The data from the  $^{13}\text{C}$ -nmr spectrum of the compound is presented in Table 33 together with the comparable data for salbutamol itself. The similarity between the salbutamol and degradation product signals suggests that most of the molecule has undergone little change.

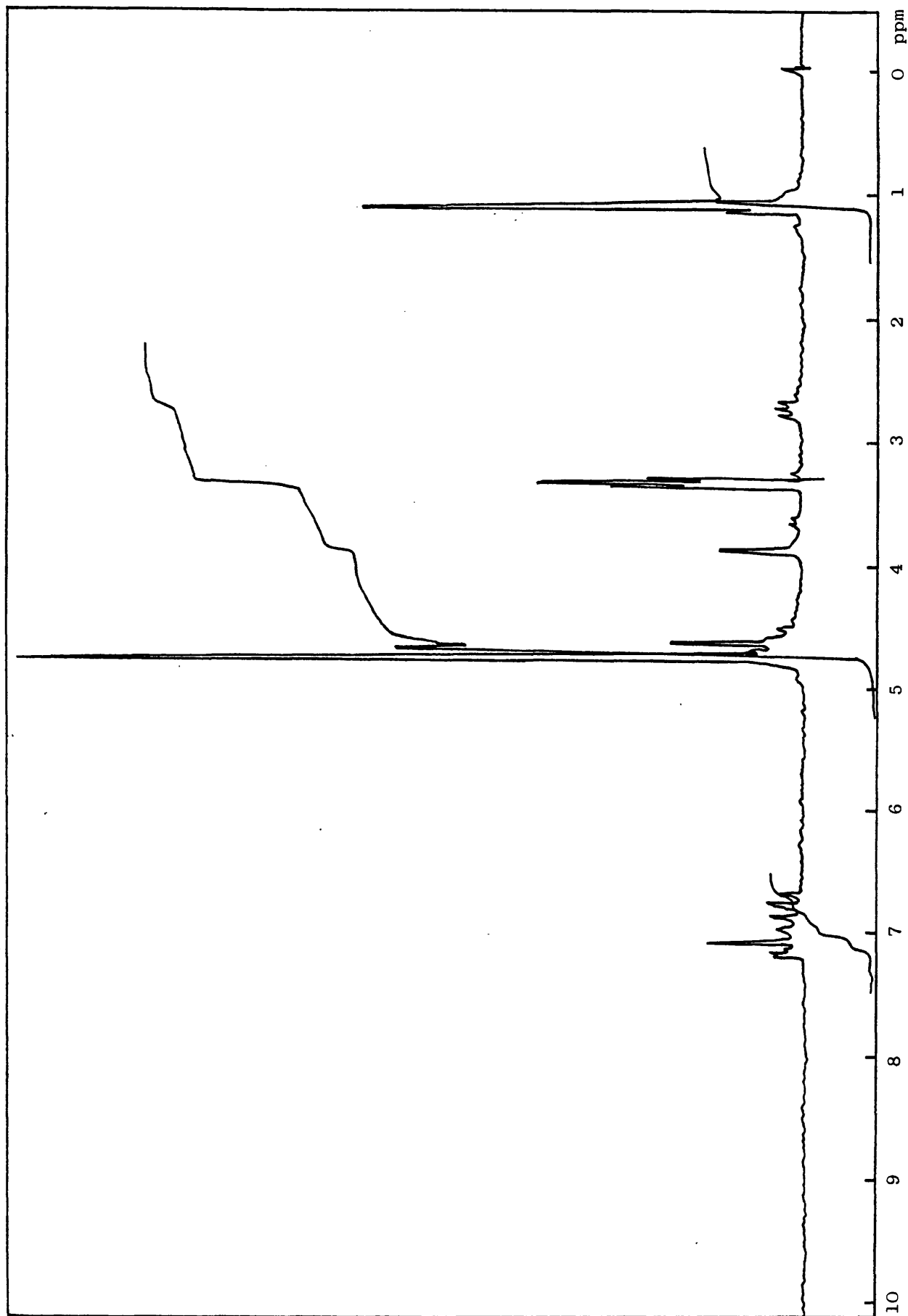
The small quantity of, possibly impure, material which was available precluded a detailed study but some conclusions as to the nature of the compound can be drawn. The increase in the number of aromatic protons and carbon atoms with retention of a single t-butyl group suggests an alkylation by one salbutamol molecule with complete loss of one side chain. The mechanistic interpretation of the loss of the side chain is problematical although hydramine fission would remove the amino group.



A structure consistent with the available data is shown in Fig. 55.



Fig. 54. The  $^1\text{H}$ -nmr spectrum of the major breakdown product of salbutamol sulphate in methanol- $\text{d}_4$  with TMS internal standard.



| Chemical shift<br>ppm (degradation product) | Chemical shift<br>ppm (Salbutamol) |
|---|------------------------------------|
| 153.7                                       | 153.3                              |
| 152.1                                       |                                    |
| 134.6                                       | 134.4                              |
| 131.3                                       |                                    |
| 127.8                                       | 127.8                              |
| 127.4                                       | 125.1                              |
| 124.4                                       | 124.9                              |
| 114.5                                       | 114.1                              |
| 114.4                                       |                                    |
| 72.1  | 72.3                               |
| 58.4  | 58.5                               |
| 50.4  | 50.5                               |
| 48.1  | 49.6                               |
| 42.0  |                                    |
| 28.7  | 28.7                               |

Table 33.  $^{13}\text{C}$ -nmr data for salbutamol and its principal degradation product. Spectra run in  $\text{DMSO-d}_6$  with TMS as internal standard.

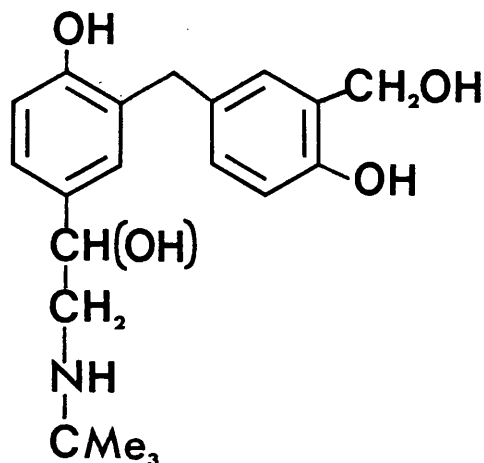


Fig. 55. Possible structure of major degradation product of salbutamol sulphate in simple aqueous solution.

The signal at  $\delta 3.8$  in the proton spectrum (Fig. 54) and at  $\delta 42.0$  in the  $^{13}\text{C}$  spectrum (Table 33) would be due to the methylene bridge between the two aromatic rings. This is in accordance with data for di-(4-methoxyphenyl) methane which has a value of  $\delta 40.0$  for the carbon resonance (139). Application of Shoolery's rule (Equation 13) gives a value of 3.98 for the proton resonance at this carbon atom (138).

$$\delta = 0.28 + \sum \sigma_{\text{eff}} \quad (13)$$

where  $\delta$  = chemical shift of substituted methylene group and  $\sigma_{\text{eff}}$  is Shoolery's constant for substituents. The slight shifts in the positions of the remaining peaks in the  $^1\text{H}$  spectrum compared to those in salbutamol are probably attributable to the change in the solvent used. This change was necessary to enable the compound to be recovered later, methanol being volatile whilst DMSO is not.

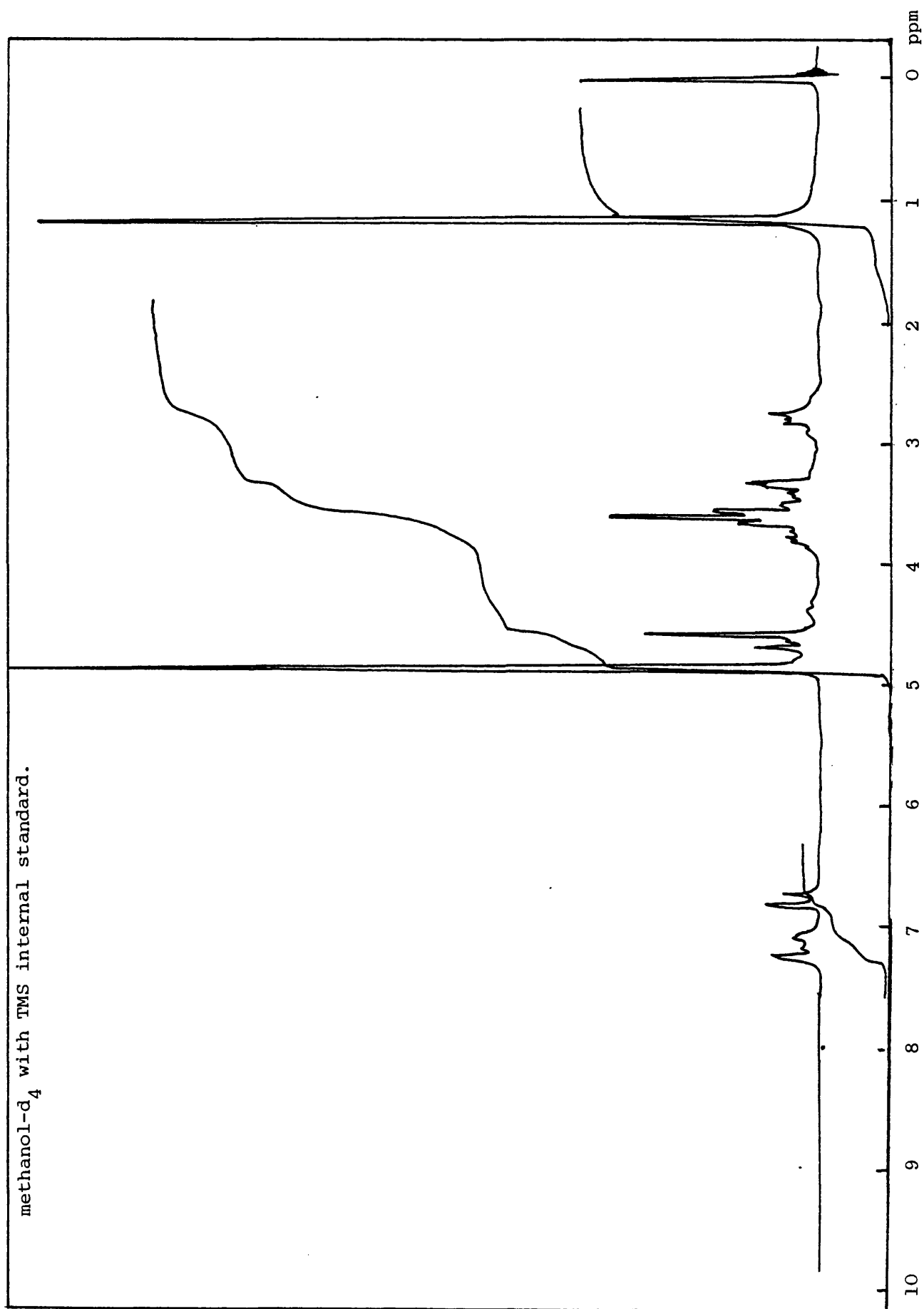
The mass spectrum of the compound was too complex for immediate interpretation and it is likely that the compound was insufficiently pure for this technique to provide useful information. There was no significant peak at  $m/e$  345, the molecular ion ( $M^+$ ) of the proposed structure, but a signal was present at  $m/e$  327 which may represent  $M-H_2O$ . Other fragmentation peaks could not be assigned with any degree of certainty.

### 3.3 The glycerol-salbutamol reaction product

The  $^1H$ -nmr spectrum of the product of the reaction between glycerol and salbutamol sulphate is shown in Fig. 56, where it can be seen that the most striking difference is in the region  $\delta$ 3.2-4.0. The integration of this group of peaks suggests an increase in the number of aliphatic protons. This fits the concept of a simple addition of glycerol, since the remaining peaks closely resemble those of salbutamol (Fig. 53).

Lack of compound prevented a well resolved  $^{13}C$  spectrum being obtained, but the available data is presented in Table 34. There appears to be an increase in the number of aromatic carbon atoms together with an increase in signals around 62 - 81 ppm. Signals from the glycerol framework would be expected to appear in this region but there appear to be more than were indicated by the proton spectrum, perhaps due to interaction between glycerol and a salbutamol degradation product of the type proposed in Section 3.2.

Fig. 56. The  $^1\text{H}$ -nmr spectrum of the product of the reaction between salbutamol and glycerol. Spectrum run in methanol- $\text{d}_4$  with TMS internal standard.



| Chemical shift<br>ppm                                      | Assignment                 |
|--|----------------------------|
| 157.1  | Aromatic?                  |
| 134.2, 130.8, 129.2<br>128.6, 127.9, 125.7<br>125.4, 116.9 | Aromatic                   |
| 81.5   |                            |
| 72.5   |                            |
| 72.2   |                            |
| 71.8   |                            |
| 69.8   |                            |
| 64.2   |                            |
| 62.1   |                            |
| 28.0   | $-\text{C}(\text{CH}_3)_3$ |

Table 34.  $^{13}\text{C}$ -nmr data for the product of the reaction between glycerol and salbutamol. Spectrum run in  $\text{d}_4$ -methanol with TMS internal standard.

The compound is very similar to salbutamol in polarity, its HPLC capacity ratio being 1.75 compared to 1.8 for salbutamol. However, this does not offer much assistance in assigning a structure to the compound.

The most likely point of attack would appear to be the nitrogen atom in salbutamol, leading to formation of a glycosidic compound or a Schiff base. This, in turn, could cyclise to produce a tetrahydroisoquinoline derivative (Fig. 57).

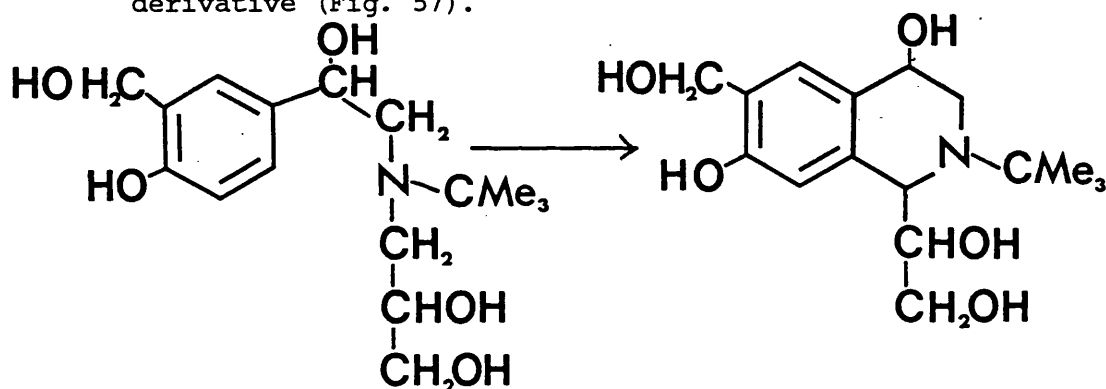


Fig. 57. Possible structure and further degradation of the glycerol-salbutamol reaction product.

The main problem with such a scheme, apart from the fact that neither structure accounts satisfactorily for all the signals in the nmr spectra, is that the ring closure has to take place meta to the phenolic hydroxyl. This is not an activated position, such closures generally occurring ortho or para to the phenolic group.

A further complication arises from the instability of the compound. Its solution darkened considerably on standing and whilst the <sup>1</sup>H-spectrum could be run within

a few minutes, the  $^{13}\text{C}$  spectrum required a long accumulation time and so could have been significantly affected by degradation of the compound. As a result, it is impossible to unequivocally assign a structure to the compound without much more data.



## Section 4.

DISCUSSION4.1 Assay Technique

Pharmaceutical stability studies may be approached qualitatively or quantitatively, the method chosen being largely dependent on the analytical techniques available. Qualitative studies involve the monitoring and identification of breakdown products but actual measurement of the extent of degradation is limited, often employing techniques such as TLC-densitometry. In contrast, when a reliable, specific assay for the compound in question is available, a quantitative approach may be adopted. Although breakdown products still need to be identified to confirm that toxic compounds will not accumulate in the formulation on storage, the principal method of assessing stability samples in this case is by assay of the active components.

In this study, a stability indicating assay was developed with the aim of taking the second approach. Attempts were made to isolate and identify the major breakdown products in order to determine the degradative pathway of the drug. Unfortunately these compounds proved to be rather labile and appreciable breakdown occurred during the isolation processes so that definitive identification of any one compound was not possible (Section 3).

The validity of using the HPLC technique for stability studies was tested initially by injecting closely related compounds such as AH6368 and isoprenaline and measuring their capacity ratios ( $k'$ ). All the compounds tested

(Table 4) were separated from salbutamol, even though in some cases the structural differences were small (Fig. 18). The internal standard, AH4045, which differs only in one ring substituent was fully resolved from the drug (Fig. 21). This suggested that breakdown products would also be well separated from the intact drug but in order to confirm this, the major degradation products were separated by preparative TLC (Section 2.2). Since these compounds were rather labile (Section 3), they were not isolated in a dry state but maintained in solution and injected onto the HPLC column immediately after elution from the TLC silica. All those which gave a significant peak in the HPLC chromatogram at the usual analytical sensitivity, eluted well away from salbutamol (Fig. 22). It was possible, by increasing the detector sensitivity by a factor of 4, to detect small peaks in some of the other samples and in a few cases, these coincided with the position of salbutamol in the chromatogram. However, since the salbutamol peak would be completely off-scale at such a sensitivity, these compounds did not present a problem in the assay of the drug by this technique. The method was therefore accepted as stability indicating and was used to assay the samples from stability experiments.

It appeared that the more polar compounds eluted fastest from the HPLC column, AH4045 and "benzyl-salbutamol" having longer retention times than salbutamol itself. However, it was not possible to directly correlate  $R_f$

values from TLC with  $k'$  values from HPLC, as demonstrated in Table 35.

| Compound                   | Rf (20°) | $k'$ |
|----------------------------|----------|------|
| Salbutamol-glycerol adduct | 0.42     | 1.75 |
| Salbutamol                 | 0.60     | 1.8  |
| Major degradation product  | 0.75     | 4.8  |
| Salbutamol-glucose adduct  | 0.85     | 0.40 |
| AH4045                     | 0.9      | 4.1  |

Table 35. TLC and HPLC parameters of salbutamol and related compounds.

This lack of correlation is not really surprising as the separation mechanisms operating in the two systems are completely different. If it had proved possible to develop a satisfactory separation on a silica HPLC column, correlation between TLC and HPLC may have been possible as claimed by some workers (101, 102). However, the differences in the chromatographic conditions make such generalisations unreliable. Equilibrium is seldom achieved in TLC and so the solvent composition alters as it progresses up the plate, resulting in variation in separation efficiency. A well conditioned HPLC column, however, should be uniform in its efficiency throughout its length. TLC is also much more sensitive to temperature fluctuations. The running speed and hence the separation of salbutamol

solutions changed considerably with the ambient temperature as demonstrated in Table 16. The HPLC separation was not sensitive to temperature and although temperature control with a water-jacketed column was tested, it did not affect the separation or capacity values of the solutes.

The principal difference between the two chromatographic systems lay in the number of degradation products detected. Most HPLC traces showed the presence of only one or two breakdown products whilst TLC detected six or more in extensively degraded solutions. This demonstrates a potential pitfall in the interpretation of analytical results. Taken alone, the HPLC results would suggest a simple degradation pathway leading to the production of one or two new compounds. Elucidation of the breakdown mechanism of salbutamol sulphate might therefore be expected to be relatively straightforward. By contrast, the TLC plates of degraded salbutamol solutions reveal such a complex pattern of compounds that development of a specific assay for the intact drug might be deemed almost impossible. In addition, degradation might qualitatively appear to be more extensive than it really is since many of the detected compounds are present in only very low concentrations. Both techniques are obviously of great value but the significance of the data they produce must be assessed with care.

The reason for the difference between the chromatograms obtained from the two techniques, as used in this study,

is two-fold. The preliminary separation by ion-exchange chromatography removes several compounds prior to injection onto the HPLC column, as demonstrated by the HPLC traces in Figure 20. The other factor relates to the high sensitivity of the colorimetric spray reagent used to visualise developed thin layer chromatograms. This is able to detect compounds at extremely low concentrations as was shown during the verification of the HPLC assay. Although several breakdown products were detected by the spray reagent and eluted from preparative TLC plates, some did not produce recognisable peaks in the HPLC chromatogram unless the sensitivity of the detector was increased by a factor of 4 to its maximum. Even at this sensitivity, the resultant peaks were very small and almost lost in the background "noise". It is possible that some of these compounds had different absorption spectra from salbutamol and could have been detected at a different wavelength. In order to check this, an approximately 50% degraded solution of salbutamol sulphate was prepared for assay in the usual way (Section 2.3) and injected onto the HPLC column. The detection wavelength was increased by 5 nm between injections and in this way the solution was scanned between 240 nm and 290 nm. There was no noticeable change in the number of peaks in the chromatogram, or in their relative intensity, and so it is unlikely that the HPLC assay "missed" compounds due to their having different absorption spectra.

The value of having two separation techniques was

demonstrated very clearly when salbutamol degradation in the presence of sugars was studied. The monosaccharides glucose and fructose formed a compound with salbutamol which ran on TLC with an  $R_f$  value, at  $20^{\circ}$ , of about 0.85. On HPLC the compound had a short retention time, appearing soon after the solvent peak and before salbutamol, suggesting that it was considerably more polar than salbutamol. Similar experiments with the polyhydric sorbitol, glycerol and propylene glycol revealed an interesting trend. Sorbitol produced a compound which behaved very similarly to that produced by glucose and fructose, whilst propylene glycol formed an adduct which eluted on HPLC between salbutamol and AH4045. Initial experiments with glycerol suggested that no reaction had occurred because no additional peak appeared in the chromatogram. This seemed unlikely in view of the close structural relationship of the three excipients. Since sorbitol, with six hydroxyl groups gave a compound with a capacity value of 1.0 and propylene glycol, with only two hydroxyls resulted in a compound with the larger value of 2.75, by interpolation, the three hydroxyls of glycerol should result in an intermediate capacity value which could coincide with that of salbutamol ( $k' = 1.8$ ). TLC examination of the solution confirmed the presence of an additional compound, well separated from salbutamol ( $R_f$  0.60) at an  $R_f$  of about 0.42 (Fig. 58). The use of a more efficient column having 1350 plates instead of the original 500 enabled the two compounds to be resolved sufficiently for the integrator to measure individual peak areas.

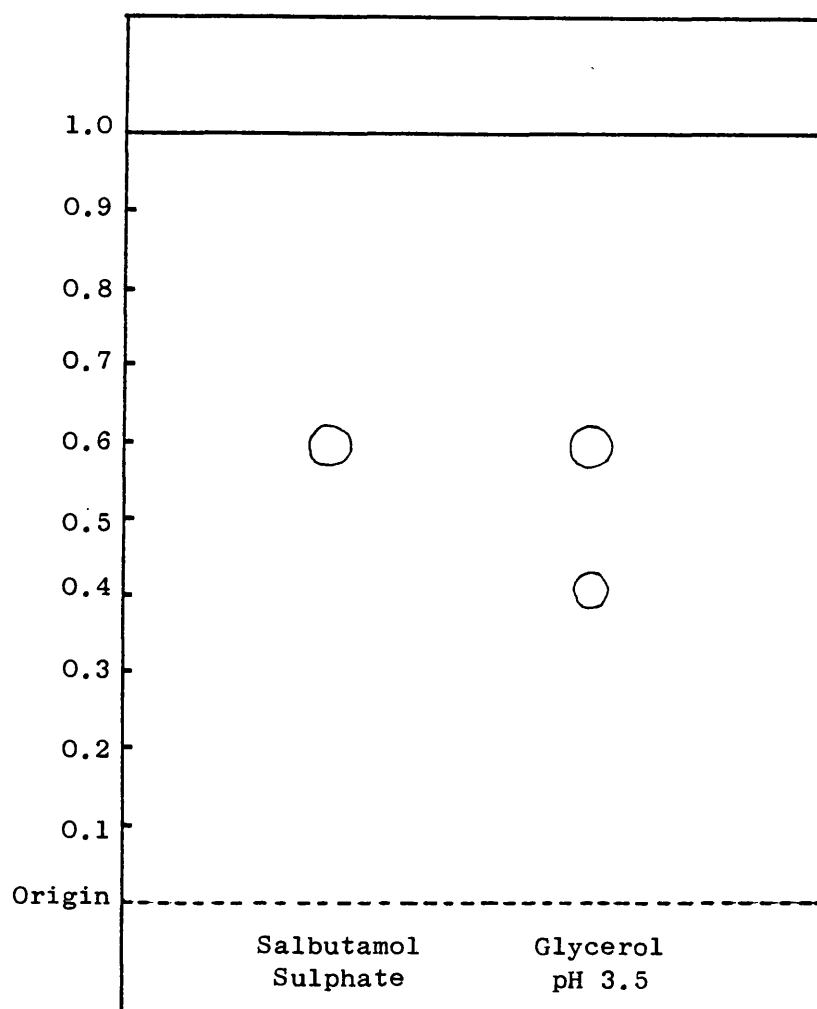


Fig. 58. Diagrammatic TLC plate of a standard 0.5% salbutamol sulphate solution, together with a sample degraded for approximately 500 hours at pH 3.5 and 70° in the presence of 50% glycerol.

The preliminary work using simple degraded solutions of salbutamol sulphate was therefore unable to predict the analytical problems which would arise on the addition of other substances. It is therefore essential to reassess all analytical techniques regularly in the light of additional information.

A good example of the need to update assay procedures to incorporate new developments is provided by the contrasting results obtained by the indoaniline and HPLC methods. The colorimetric reaction evidently assays a considerable proportion of the degradation products as salbutamol, since the results from this method were significantly higher than those obtained by the chromatographic technique (Tables 12 - 15, Figs. 25 - 27) and the same spectrum of breakdown was visualised on TLC plates by both the indoaniline and MBTH spray reagents. The reason for this overestimation by the indoaniline reaction lies in the fact that modifications to the amino side-chain of salbutamol would result in the same indoaniline dye since this portion of the molecule is "lost" during the reaction (Fig. 11). An additional problem arises from the fact that although a chemically different dye will result from a change in the ring substitution of the drug, the absorption spectrum of this dye is likely to overlap that of the dye formed from salbutamol. Overestimation of the salbutamol content of degraded solutions is therefore inevitable.



It is interesting to note that the difference in assay results between the two techniques varies with the degradative conditions. This is summarised in Table 36 which gives the colorimetric results equivalent to an HPLC assay of 57% under various conditions. This data is taken from

| Conditions   | HPLC | Colorimetric |
|--------------|------|--------------|
| 0.1% pH 9.0  | 57%  | 70%          |
| 0.5% pH 7.0  | 57%  | 89%          |
| 0.05% pH 3.5 | 57%  | 69%          |

Table 36. Comparison of assay methods for salbutamol sulphate.

Table 15 and Figs. 25 and 26. It is apparent that the discrepancy between the two methods is greatest at the highest concentration but it is likely that the pH differences also have an effect. The differences between the two sets of results suggest a change in the degradation products under varying conditions although TLC examination did not detect significant changes in the breakdown pattern with pH (Fig. 41) or concentration. However, since it is difficult to estimate the intensity of TLC spots visually, it is possible that there were some changes in the relative proportions of some degradation products which were not observed.

A further consideration is that the colorimetric method is laborious and time-consuming whilst the HPLC method, in spite of the preliminary sample preparation is cleaner and quicker, allowing replicates to be performed more easily. It would probably be possible to modify the system to incorporate an ion-exchange pre-column, enabling sample preparation and assay to be carried out in one operation. A greater through-put of samples could also be achieved with an autoinjector.

The reproducibility of the HPLC assay proved satisfactory within any one day, replicate injections usually having a coefficient of variation of less than 1.0%. The only exception to this was the first injection of a new solution which often gave an anomalous result. The peak area ratio of this injection was often high if the previous solution had been more concentrated or low if more dilute (Table 9).and this suggested inefficient rinsing of the syringe between samples. In order to correct this, the syringe and loop were rinsed four times with distilled water and four times with the new solution before making an injection but this did not improve the result. As a consequence, the result for the first injection of a new solution was always ignored. The reason for this anomaly is not clear but would appear to be a function of the column itself. It is possible that a certain percentage of any injection is retained by the column, a new injection displacing the retained portion

of the previous one. The percentage must be the same for each solution since the calibration lines were linear and passed through the origin.

Table 5 shows that the reproducibility of a single determination tended to improve with increasing concentration. The most dilute solution (0.02% salbutamol sulphate) gave results with a coefficient of variation, over six determinations, of 1.36%. The more concentrated solutions usually showed a variation of less than 1.0% and often less than 0.5%. This is a very acceptable level of reproducibility for this type of assay procedure. The increase in variability with falling concentration tends to provide the lower limit for the assay. In this study, 0.02% solutions were the most dilute to be used routinely. This refers to the concentration before Sephadex treatment which diluted the samples by a factor of 5. The assay therefore detected 0.04 mg salbutamol sulphate per ml (0.4  $\mu$ g in 10  $\mu$ l sample) as used routinely. More dilute samples could be processed by increasing the detector sensitivity enabling 0.1  $\mu$ g to be detected, or by increasing the volume applied to the Sephadex column. The columns could handle at least 2 mg salbutamol sulphate and so this could be applied in as large a volume as necessary and still be eluted into 10 ml. This would enable the system to be used on the same settings for a wide range of sample concentrations.

The daily variation in the column performance (Table 6)

necessitated daily calibrations. Three standard solutions were used to ensure that the calibration was linear and these were interspersed throughout the day's samples. No drift in the calibration was found over the course of a day, since standards injected at the beginning and end of the day gave the same result (Table 8). Similarly, standards were not assayed in any particular order yet always gave a line which had a standard deviation to slope ratio of less than 3%. The variation in the slope of the calibration line was random over the course of a year (Appendix 2) and no pattern could be detected. The mean value of the slope obtained from the data in Appendix 2, is 9.495 with a coefficient of variation of 6.1%. This is in close agreement with the mean of the values in Table 6 which is 9.43 with a coefficient of variation of 6.6%, even though the latter data arises from 5 point calibrations instead of 3 point. This serves to confirm that the variation is truly random with no apparent long term trend. The effect was not temperature dependent because use of a water-jacket to control the column temperature did not improve the reproducibility. The drift must be related to the conditions in the column when no solvent is flowing through it, since changes only took place when left, for example, overnight and not when used over a similar period. The packing material may settle or expand, due to relaxation, during this time or accumulated contamination may elute whilst conditioning the column for a day's analysis. The randomness of the phenomenon seems to rule out progressive breakdown of the column.

#### 4.2 Authenticity of kinetic data

The overall reproducibility of the kinetic experiments was dependent largely on the reliability of the assay and the stability and accuracy of the pH stat equipment. Although most pH electrodes can be used at temperatures up to about 70<sup>o</sup>, they are stable at elevated temperatures for only a few minutes and so are unsuitable for long term pH monitoring as required by the pH-statting technique used in these studies. Most experiments lasted for several weeks and so pH drift had to be minimal. Special high temperature electrodes were therefore used which have a large bulbous reservoir of electrolyte. The large surface area of this reservoir makes it a potential source of considerable heat loss which could result in a temperature gradient along the length of the electrode. This, in turn, could produce erroneous fluctuating readings and so the electrode bulb and stem were lagged to minimise heat losses. In fact, identical pH readings were obtained with unlagged electrodes but lagging was used to reduce the possibility of pH drift due to draughts during the course of an experiment.

A further complication arose from the nature of degraded salbutamol sulphate solutions. As breakdown of the drug proceeded, the electrodes often became coated with a brownish deposit which could potentially affect their response. However, when checked with standard buffers at the end of an experiment, they were found to be accurate to within  $\pm 0.05$  pH units (see

p.93 ). Addition of titrant was therefore initiated by real pH changes ensuing from degradation of the drug and not instrumental drift.

The reproducibility of the kinetic experiments was established at the outset of the work using 2.0% salbutamol sulphate solutions at pH 9.0 and 70° (Table 18). The mean value of the apparent first order rate constant from these three experiments was  $9.94 \times 10^{-6} \text{ s}^{-1}$  with a coefficient of variation of 1.4%. This represents good reproducibility and so the experimental method was believed to be reliable. Under the conditions used for this initial study, results could be obtained quickly but most of the subsequent work used more dilute solutions and milder conditions and so experiments often took several weeks to complete. In the extreme case, one experiment at pH 3.0 and 70° lasted nearly 10 months and still achieved only 20% degradation. It was therefore impractical to repeat every experiment if the time and equipment were to be used to the best advantage.

A further difficulty lay in the shape of the graphs obtained from many of the kinetic experiments. Whilst first order plots from simple solutions were essentially linear, non-linear results were obtained from most of the experiments which contained additives (e.g. Figs. 43, 45, 51). This makes it much more difficult to reproduce the graph because the exact shape will be dependent on the sampling times. In addition, when apparent first

order rate constants are calculated from the initial, linear, portion of such graphs, the value obtained will be strongly dependent on the number of data points used in the regression analysis. The number was always decided by visual assessment of the data but consistent results were obtained as demonstrated by the data for citrate-containing solutions (Section 2.12f, Table 25, Fig. 43).

The reproducibility of the experimental data was confirmed by performing replicates of a few experiments at intervals throughout the work. The results are summarised in Table 37. Although the experiments for the first reproducibility study at pH 9 were all performed within one month, the time interval between some replicates was considerable and yet consistent results were still obtained. Figure 43 demonstrates the reproducibility of a distinctly non-linear result, the replicates in this case being performed two months apart. The statistical significance of the variation between replicates shown in Table 37 was tested in all cases by the student t-test (Appendix 1). In no case were the differences significant at the 5% probability level.

Further support for the authenticity of the kinetic results was provided by the Arrhenius plots (Fig. 39). The Arrhenius results at pH 9 show good linearity and whilst those obtained at pH 7 have a lower linear correlation coefficient, the deviations do not suggest major experimental errors.

| Salbutamol sulphate<br>Concentration % w/v | Conditions              | Time interval | $k(s^{-1})$  | Std. Dev.   |
|--|-------------------------|---------------|--|---|
| 2%   | pH 9.0, 70°             | 2 weeks       | 9.89 x 10 <sup>-6</sup><br>10.14 x 10 <sup>-5</sup><br>9.83 x 10 <sup>-6</sup> | 1.98 x 10 <sup>-7</sup><br>8.31 x 10 <sup>-8</sup><br>3.97 x 10 <sup>-7</sup> |
| 0.5%                                       | pH 9.0, 70°             | 5 months      | 7.00 x 10 <sup>-6</sup><br>6.81 x 10 <sup>-6</sup>                             | 7.78 x 10 <sup>-8</sup><br>6.22 x 10 <sup>-8</sup>                            |
| 0.5%                                       | pH 7.0, 70°             | 1 month       | 9.94 x 10 <sup>-7</sup><br>10.75 x 10 <sup>-7</sup>                            | 3.50 x 10 <sup>-8</sup><br>3.92 x 10 <sup>-8</sup>                            |
| 0.5%                                       | pH 7, 70°, 0.1M citrate | 2 months      | 1.03 x 10 <sup>-6</sup><br>1.11 x 10 <sup>-6</sup>                             | 3.67 x 10 <sup>-8</sup><br>7.67 x 10 <sup>-8</sup>                            |
| 0.5%                                       | pH 7, 70°, 0.05% EDTA   | 4 months      | 1.43 x 10 <sup>-6</sup><br>1.51 x 10 <sup>-6</sup>                             | 1.18 x 10 <sup>-7</sup><br>5.47 x 10 <sup>-8</sup>                            |

Table 37. Reproducibility of apparent first order rate constants for the degradation of salbutamol sulphate solutions under various conditions.



A potential source of error lay in the preparation of the salbutamol sulphate solution at the beginning of a kinetic run. The majority of the liquid was pre-equilibrated at the experimental temperature and then the required amount of drug was added as a concentrated solution. It was not possible to pre-equilibrate this solution because of the danger of initiating degradation too soon. This was a real problem because concentrated solutions were more susceptible to degradation than dilute ones (Fig. 36, Table 20). The long duration of each experiment meant that it was not practical to keep a concentrated stock solution because it would not be used within a realistic time and so would have to be freshly prepared for each experiment. Therefore in order to avoid unnecessary wastage of the drug, the exact amount needed for each particular experiment was weighed into a 10 ml volumetric flask and dissolved in water up to volume. The resultant solution was added to the reaction vessel and complete transference of the drug was ensured by rinsing the flask with measured volumes of distilled water to bring the total in the reaction vessel to the required volume. Assay of the initial sample, which was withdrawn as soon as possible, confirmed the concentration of the reaction solution. Apart from a few early experiments, the measured starting concentration was always within  $\pm 4\%$  of the theoretical value.

#### 4.3 General factors affecting salbutamol sulphate stability

The kinetic studies commenced with a series of experiments designed to determine the major factors affecting the stability of salbutamol sulphate solutions. This enabled later work to be performed under controlled conditions of concentration, temperature, pH etc. so selected that the maximum amount of relevant data could be collected in the time available.

Oxygen was soon shown to have a profound effect on the degradation of the drug. Solutions which were bubbled with the gas at 10 - 15 ml per minute followed apparent first order kinetics, semi-logarithmic plots being linear, unlike those obtained from unbubbled solutions which were sigmoidal (Fig. 34). The slopes of the lines from oxygenated experiments were approximately 30% greater than the slope of the linear portion of the sigmoidal graphs. Increasing the oxygen flow rate to 50 ml per minute had no effect on the rate of salbutamol breakdown (Table 19) and so the solution must be effectively saturated with oxygen at the lower flow rate. It did not prove possible to use a sintered glass "bubbler" as the gas inlet because of the shortage of space within the flask. However, these experiments suggest that adequate gas transfer was able to take place even at the lower flow rate, using a fine glass tube as the inlet.

Since the oxygen flow rate was not critical, the exact rate used in each experiment was not measured

precisely but always lay in the range 10 - 15 ml per minute as assessed by the bubbling rate. A higher rate was used in sugar solutions than in simple drug solutions because of their increased viscosity, which tended to hinder efficient mixing of the pH-stat titrant. The increase in agitation produced by a faster flow of gas improved the efficiency of the pH-stat control.

The importance of oxygen in the degradative mechanisms of salbutamol sulphate was demonstrated by the experiment in which high purity nitrogen replaced oxygen as the bubbled gas (Fig. 35, Table 19). The rate of breakdown was about three times slower than that under oxygen although it was still appreciable, the drug having a half-life of 70 hours at pH 9 compared to 25 hours under oxygen. The breakdown pattern observed on TLC plates was the same as that in oxygenated solutions and this implies that the degradation was due to incomplete removal of oxygen from the system. "White spot" nitrogen is claimed to contain no more than 5 ppm oxygen and this is unlikely to account for the degradation rate observed. However, if the solution contained residual oxygen when the drug was added at the beginning of the experiment, this could have been the cause of the breakdown. If this was the case, a fall in the rate constant towards the end of the experiment would be expected because the limited amount of oxygen would be used up in the reaction; such an effect was not observed. The most likely explanation

is that small amounts of oxygen, either residual in the solution or introduced by the traces in high-purity nitrogen, initiate autoxidation reactions. These are mediated by free radicals and are self-propagating, even when the initiator is no longer present. Catecholamines have been reported to be subject to autoxidation in the absence of oxygen. For example, adrenaline solutions stored under nitrogen have been shown to polymerise to form "adrenaline-black" or melanin (112), trace amounts of oxygen probably initiating the reaction. Free radical mechanisms have frequently been postulated for catecholamine oxidations (28, 30-32, 37, 110) and although salbutamol lacks the ortho-hydroxyl groups of a catechol, radical formation by the phenol would not be impossible. In oxygenated solutions the rate of reaction would be higher because initiation could take place constantly, but the reaction pathway would be the same, producing the same spectrum of breakdown products.

The concentration of the drug itself was found to be another major influence on the rate of degradation. Since oxidation reactions often appear to follow zero-order kinetics, dilute solutions are often stated to be more susceptible to such degradation than concentrated ones (23), because the percentage loss is greater. However, in the case of salbutamol, the more concentrated solutions were less stable than the dilute ones, as shown in Figure 36 and Table 20, where it can be seen that the apparent first order rate constant increases with concentration. The

implication is that oxidation is not the sole mechanism operating in the breakdown process and that although oxygen plays an important role in the degradation of the drug, it is not the rate limiting factor (Table 19).

It was also possible, since salbutamol is an ionisable molecule, that this concentration effect was simply due to changes in the ionic strength of the solution. Ionic strength has been shown to affect the degradation of several drugs (131-135) although in most cases the reactions concerned are hydrolytic. However there are a few examples of non hydrolytic mechanisms being influenced by ionic strength, an example of which is the degradation of chlorpromazine which is believed to be oxidative and mediated by free radicals (133). Experiments using a low salbutamol sulphate concentration at high ionic strength proved that this was not the important factor in this case (Fig. 38, Table 21) and that therefore the increased degradation rate in concentrated solutions is a function of the increased number of drug molecules present.

When the data were plotted according to the half-life method for the determination of reaction order (Equation 7), the graph obtained (Fig. 37) was not completely linear, the half-life of the drug in 0.1% solution being approximately 10 hours (16%) greater than would be predicted from the other three data points. Because of this, least squares regression analysis was applied to both the total data and to the data omitting

the 0.1% result. The total data gave a reaction order of 1.37 whereas that omitting the 0.1% data point gave an order of 1.3. In either case, the result confirms that the degradation is a complex process, probably involving several parallel, consecutive or opposing reactions (24-26). Experiments using drug concentrations such as 0.2% and 0.4% would help to fill the gap in the half-life plot and determine whether there is a discontinuity. If there is, the fall in reaction rate in dilute solutions emphasises the importance of intermolecular reactions between drug molecules rather than solute-drug interactions.

Salbutamol has two functional groups which are capable of ionisation. These are the phenolic hydroxyl and the secondary amine and their  $pK_a$ s at 25° are 9.35 and 10.3 respectively (12). The three ionic species are shown in Figure 59 where it can be seen that salbutamol is always ionised, regardless of pH, due to the existence of a zwitterionic form. The proportion of each species present in solution at any given pH is depicted in Figure 60, where the shaded portion represents the region of the zwitterion. Although the  $pK_a$ s are likely to shift with temperature, analogy with results published for pirbuterol (42) suggests that the phenolic  $pK_a$  will not alter significantly between 25° and 70° and although the amino  $pK_a$  may fall by up to one pH unit, this will not result in the formation of a neutral species.

The pH stability profile (Fig. 40) shows that the rate

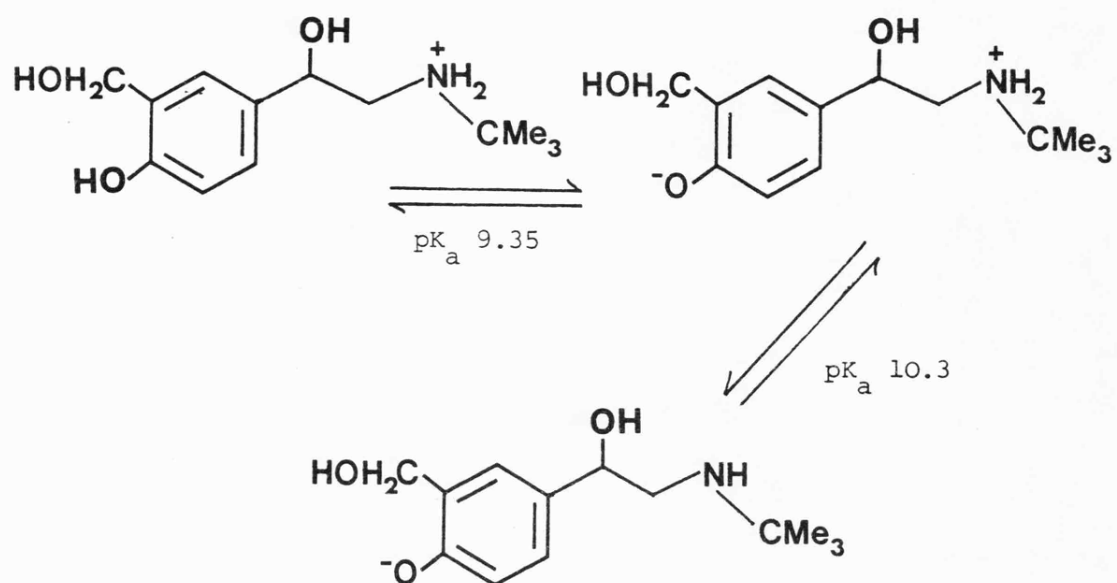


Fig. 59. The ionisation states of salbutamol

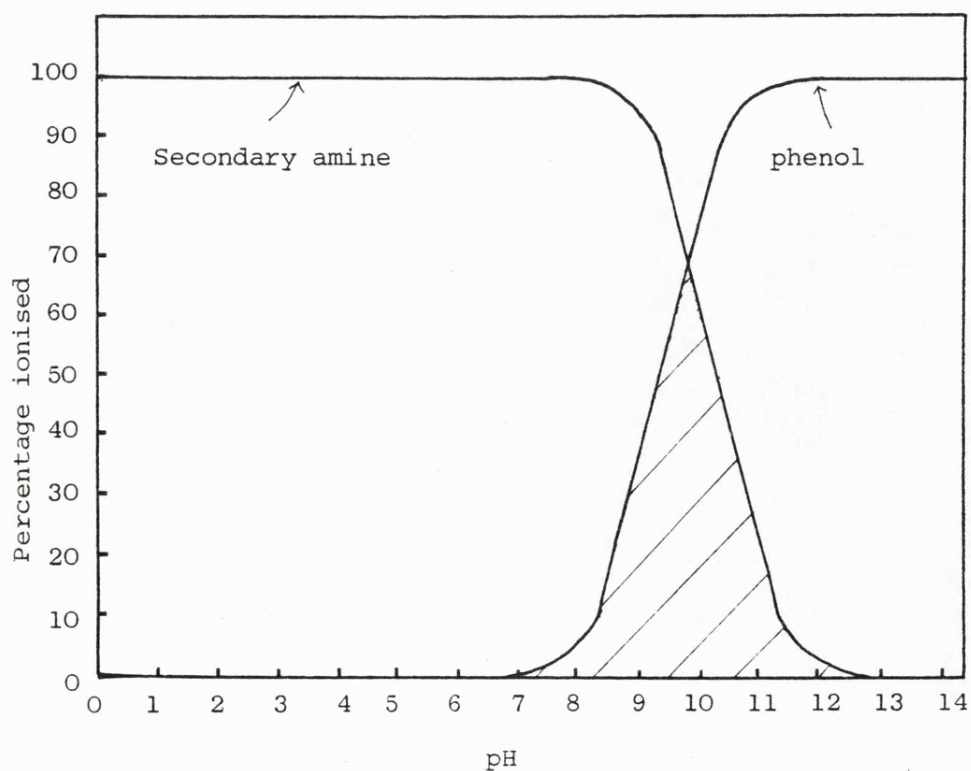


Fig. 60. The ionisation of salbutamol, the shaded area representing the zwitterionic form.

of salbutamol degradation is greatest in the region of pH 9. This coincides with the region of maximum concentration of zwitterion (Fig. 60) and therefore it may be inferred that the zwitterion is the least stable species. This conclusion is in agreement with that of Bansal and Monkhouse (42) who examined the stability of pirbuterol, the pyridine analogue of salbutamol. The drug exhibited its lowest stability in the pH range 8-9, where, like salbutamol, it is zwitterionic.

Figure 40 shows that the relationship between  $\log k$  and pH is approximately linear between pH 5 and pH 8. However, the slope of this line is about 0.6 and so the degradation mechanism is not a simple base catalysed reaction which would have a slope of one. The maximum at pH 9 is believed to be real because the  $k$  value at pH 10 represents a 25% reduction on that at pH 9 and this is well outside the normal experimental variation in replicate studies (Table 37).

Although Figure 40 shows that salbutamol is most stable in the pH range 3-4, it was not possible to define this minimum because the rate of reaction was so slow that no detectable breakdown occurred during three months storage at pH 3.5 and 70<sup>0</sup>. The presence of this minimum cannot be explained on ionisation grounds as the molecule is fully protonated at the secondary amine in this region. Pirbuterol was not found to exhibit a minimum of this sort, the pH-profile levelling out to a plateau minimum below pH 2.



This difference can be explained if the principal mechanism of degradation for both drugs at acidic pH is by electrophilic attack. This characteristically takes place in acidic media but under these conditions the heterocyclic nitrogen atom in pyridine is protonated. This has the effect of deactivating the ring to electrophilic substitution as shown in Figure 61.

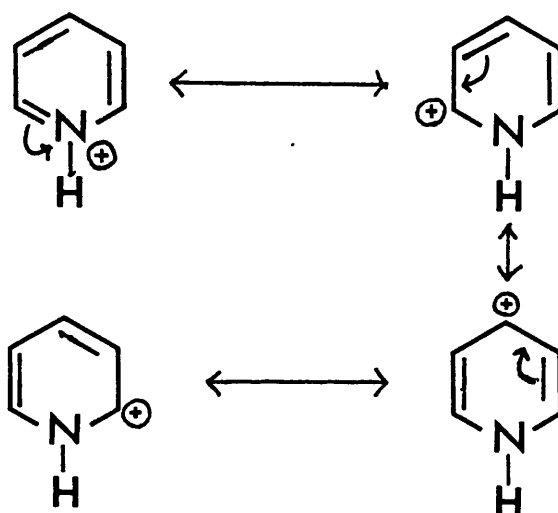


Figure 61. Stabilisation of the pyridinium ion.

The pyridine ionisation has a  $pK_a$  of about 3 in pirbuterol and so below pH 3 the drug will be extensively ionised, reducing the possibility of electrophilic attack. In contrast, the benzene ring in salbutamol is not subject to this deactivation and will be more liable to react under increasingly acidic conditions, causing an upturn in the stability profile. The slope of this portion of the graph is also far removed from unity, being about 1.5. This indicates that the degradation is not a simple hydrogen ion catalysed reaction.

The deactivating effect of the pyridine ring has a marked effect on the reactivity of pirbuterol over the whole pH range. At pH 8 and 90°, the drug has a half life of about 6 days whereas salbutamol at pH 9 (the corresponding point of maximum breakdown) and 70° has a half-life of only 28 hours. Salbutamol is also much more sensitive to pH than pirbuterol, the rate constant varying by a factor of  $10^3$  from  $8.08 \times 10^{-9} \text{ s}^{-1}$  at pH 3 to  $6.94 \times 10^{-6} \text{ s}^{-1}$  at pH 9. The corresponding range for pirbuterol spans only a 33 fold variation from  $4.47 \times 10^{-8} \text{ s}^{-1}$  at pH 1 to  $1.49 \times 10^{-6} \text{ s}^{-1}$  at pH 8.

The fact that both drugs are always ionised would be expected to make them sensitive to ionic strength effects but this is not the case. Baxsal and Monkhouse (42) report that preliminary experiments indicated that the breakdown of pirbuterol was affected by oxygen tension and buffer catalysis but not by ionic strength effects or light and similar results were obtained in this study of salbutamol. Equation (14), which is derived from the Brønsted and Bjerrum equations (24-26), predicts that at low concentrations and ionic strengths, the reaction between two ions should vary logarithmically with the square root of the ionic strength.

$$\log \frac{k}{k^0} = 1.02 z_A z_B \mu^{\frac{1}{2}} \quad (14)$$

where  $k$  = rate constant and  $k^0$  = rate constant at infinite dilution

$z_A, z_B$  = charge on ions A and B and  $\mu$  = ionic strength

At ionic strengths above 0.01 and up to 0.1, this equation is modified to equation (15)

$$\log \frac{k}{k^0} = 1.02 z_A z_B \left( \frac{\mu^{\frac{1}{2}}}{(1 + \beta \mu^{\frac{1}{2}})} \right) \quad (15)$$

where  $\beta$  is a constant, related to the ionic diameter of A and B. At even higher values of  $\mu$ ,  $\log k$  becomes proportional to ionic strength (136), even when the reacting species are uncharged.

The marked pH-dependence of the breakdown processes of salbutamol suggests the involvement of ionic attacking species ( $\text{OH}^-$  and  $\text{H}^+$ ), and taken together with the ionic nature of the drug, would predict an ionic strength effect. The fact that ionic strength changes do not influence the rate of reaction implies that uncharged species such as solvent molecules, oxygen molecules or free radicals play a very important role. Equations (14) and (15) predict that reactions involving such species will be independent of ionic strength although, in these experiments, the conditions used fall outside the accepted limits for these relationships.

The reaction order of 1.3-1.4 calculated for salbutamol would suggest the involvement of free radical mechanisms which often have non-integral orders. The complex TLC patterns provide further confirmation of such a mechanism. Free radicals are highly reactive species and can therefore initiate several different degradative processes. This would explain the large number of compounds produced during

the breakdown of salbutamol and would also account for the apparent similarity of the breakdown spectra under different conditions of pH (Fig. 41).

The oxidation of adrenaline and other catecholamines is also believed to proceed via free radicals (28, 37). In contrast, however, pirbuterol was found to follow first order kinetics (42) and did not exhibit the concentration effect of salbutamol. TLC examination of degraded solutions revealed a much simpler pattern of breakdown in the pyridine derivative, only one degradation product being detected above pH 8. The evidence of the thin layer chromatograms and the first order rate dependence suggests that pirbuterol degrades by a simple mechanism at high pH. The mechanism at pH 8 and below is different because several breakdown products are produced but the pattern, nevertheless, is much simpler than that found in degraded salbutamol solutions.

Useful information about reaction mechanisms can often be gained from the temperature-dependence data when plotted according to the Arrhenius relationship (Equation 8 ). Studies were carried out initially at pH 9.0, where the rate of salbutamol degradation is rapid, and later at pH 7.0. This latter pH was chosen because it lay on the linear portion of the pH-stability profile and was used for many of the subsequent experiments employing additives such as buffer salts and sugars. Degradation was slower than at pH 9.0 but still fast enough for results to be obtained within realistic times.

The graph at pH 9.0 displays good linearity but the one at pH 7.0 appears, on visual inspection, to be sigmoidal, the discontinuity being in the region  $70^{\circ} - 75^{\circ}$  (Fig. 39). However, least squares regression analysis of the data gives a line which is parallel to that at pH 9.0 and having a standard deviation to slope ratio of only 5.3%. Discontinuities in an Arrhenius plot usually indicate a change in reaction mechanism but the sigmoidal nature of the graph is unusual, deviations usually being in a single direction away from the expected linear relationship. The activation energies, calculated from the regression lines, were found to be  $106.84 \text{ kJ mol}^{-1}$  at pH 9.0 and  $107.22 \text{ kJ mol}^{-1}$  at pH 7.0. These are in such close agreement, both having a coefficient of variation of less than 5.5% (Table 23), that it seems justifiable to treat the plots as linear. It would be useful to confirm this conclusion by obtaining additional data at intermediate temperatures and by studying the temperature-dependence at other pH values.

The close similarity of the activation energies and the TLC patterns (Fig. 41) at both pH 7.0 and pH 9.0 implies that the reaction mechanism does not change significantly with temperature or pH over the ranges studied. However, if the degradative process consists of a complex network of reactions, as suggested by the thin layer chromatograms, marked deviations from linearity would be expected. In such a system, each reaction would have an individual activation energy and temperature changes would therefore affect each reaction to a different extent, resulting in

a change in the reaction mechanism. If the initial reaction is the rate limiting step, linear Arrhenius plots may be expected although differences in TLC patterns would probably still be apparent.

This situation is resolved if radical formation is the primary, rate limiting process under all conditions. These radicals would then be able to react further by a number of routes, producing the variety of breakdown products detected. Such a mechanism is supported by the ionic strength results (Table 21), since radical formation is unlikely to be affected by ionic strength effects. An increase in salbutamol sulphate concentration would result in greater numbers of radicals which could, in turn, explain the increased degradation rate in more concentrated solutions.

Much of the later work on formulation excipients was conducted at pH 3.5, the region of minimum salbutamol sulphate degradation (Fig. 40). It would therefore have been useful to carry out an Arrhenius study at low pH (e.g. pH 3) in order to confirm that the primary reaction is not different from that at higher pH. However, the slow rate of reaction in simple salbutamol sulphate solutions at low pH put this beyond the scope of the present work but evidence from thin layer chromatograms suggests that no major differences would be found (Fig. 41).

It is not usually advisable to conduct Arrhenius-type

studies at points of inflection on the pH-stability profile of a drug because more than one reaction mechanism is operating at this point and so the results are difficult to interpret. It is surprising that a good linear relationship was found at pH 9 in the salbutamol study and this gives additional support to the theory of a common rate limiting step under all conditions. In their study of pirbuterol, Bansal and Monkhouse (42), conducted Arrhenius studies at pH values where they predicted that a single ionic species would be present. These values coincide with inflection points in the pH profile of the drug and although they quote the activation energies so calculated, they give no indication of the linearity of the graphs obtained (Table 38).

| pH  | Activation energy<br>kJ mol <sup>-1</sup> |
|-----|---|
| 1   | 36.8                                      |
| 5   | 46.0                                      |
| 8.5 | 39.8                                      |
| 11  | 49.8                                      |

Table 38. Activation energies for 0.5% pirbuterol solutions over the temperature range 70° - 90° (42)

The activation energies for pirbuterol are significantly lower than that obtained for salbutamol (107 kJ mol<sup>-1</sup>) and again implies a difference in reaction mechanism between the two drugs, as confirmed by the difference in

TLC patterns of degraded solutions of the two drugs.

The enthalpies of activation of adrenaline and other catecholamines have been calculated by Beijersbergen van Henegouwen *et al.* (28). All were in the range 50 - 100 kJ mol<sup>-1</sup>, whilst Wall and Sunderland (41), using colorimetric and fluorimetric assays, obtained a value of 134 kJ mol<sup>-1</sup> for the activation energy of salbutamol over the temperature range 40° - 60° at both pH 2.4 and pH 7.1. This was reduced to 92 kJ mol<sup>-1</sup> at pH 8.3. They also found that the Arrhenius plots were not linear above 60° and concluded that there must be a change in the reaction mechanism. No such change was detected in this study and the activation energy was found to be the same at both pH 7.0 and 9.0. These differences are almost certainly due to the spectroscopic assay methods employed and the buffer solutions used to control the pH in the earlier study. At the lower temperatures, degradation was considerably less than 50% and, in one case, less than 10% and so the validity of the calculated rate constant is questionable. In addition to this, the first order plots, particularly at 70°, showed very marked deviations from linearity and yet the total data appears to have been used in the determination of the rate constant. It has been shown (Tables 12-15, Figs. 25-27) that the colorimetric assay seriously overestimates the salbutamol content of degraded solutions and this casts further doubt over the validity of the earlier study. However, since it has also been shown that phosphate accelerates the degradation of the drug



(Table 25), the phosphate buffers used may have counter-acted the errors in the assay!

Buffers have been found to affect the degradation of pirbuterol (42), carbuterol (44), adrenaline (45) and catecholamines (46) and so salbutamol may be expected to be similarly affected. The most common buffer effect is one of acceleration but retardation by specific interaction such as that between borate and catecholamines (46) has also been reported. The stability of salbutamol sulphate solutions in the presence of citrate, phosphate and acetate was studied, all at pH 7.0 and a buffer concentration of 0.1M. The results (Fig. 42, Table 25) reveal that acetate has a marked stabilising effect, reducing the rate constant by about 30% whilst phosphate increases it to a similar degree. Although the acetate graph was linear, the phosphate line showed some sigmoidal character and the citrate plots were clearly non-linear. Figure 43 demonstrates that this non-linearity was a reproducible effect, the reaction rates slowing down when the residual drug concentration falls to 70% and then increasing again below 60% residual drug concentration. The effect of this is that the overall rate of degradation in the presence of 0.1M citrate is not markedly different from that in simple salbutamol sulphate solutions although the initial and final rate constants were approximately 20% greater. This result is difficult to explain because the pH was controlled by the pH-stat in these experiments and so the salts did not act as true buffers. Their degree of dissociation should therefore have remained

constant and so deviations from linearity cannot be ascribed to changes in the ionic species present.

The degree of ionisation of an acid at a given pH may be calculated from its  $pK_a$  using the Henderson-Hasselbalch equation (16).

$$pH = pK_a + \log \frac{[\text{salt}]}{[\text{acid}]} \quad (16)$$

The  $pK_a$  values (119) of the acids used in these experiments, together with their dissociation at pH 7.0 are presented in Table 39.

| Acid       | $pK_a$       | Dissociation at pH 7   |
|------------|--------------|--|
| Acetic     | 4.8          | Acetate:acid (160:1)   |
| Citric     | 3.1,4.7,6.4  | Citrate <sup>3-</sup> :citrate <sup>2-</sup> (4:1)                                   |
| Phosphoric | 2.1,7.2,12.7 | HPO <sub>4</sub> <sup>3-</sup> : H <sub>2</sub> PO <sub>4</sub> <sup>-</sup> (0.6:1) |

Table 39.  $pK_a$  values of "buffer" acids and their dissociation at pH 7.

There does not appear to be a relationship between the degree of ionisation and the catalytic effect, if any, of the salt and so it must be concluded that the effects are specific to the ions concerned. The sigmoidal nature of the citrate results (Fig. 43) may be due to instability of the citrate ions. Caramelisation has been reported to occur when dextrose-citrate solutions are autoclaved (137) although this is less marked if citric acid is used instead

of sodium citrate. The discoloration is probably primarily due to the sugar but the citrate activity is also reduced. The graph obtained from the salbutamol stability experiments containing citrate could be explained if citrate degradation occurs, resulting in a slowing of the salbutamol breakdown. Further citrate decomposition could form new products which accelerate the reaction again. Whatever the explanation of these effects, the results demonstrate the dangers of using buffer solutions to control the pH in stability studies without first assessing their specific actions. The stabilising action of some buffers could be used to advantage in the formulation of liquid dosage forms, provided they are physiologically acceptable.

Phosphate has been shown to have a catalytic effect on the anaerobic breakdown of carbuterol (44), but to inhibit the manganese-catalysed degradation of catechol and related compounds (46). This latter effect was presumably due to chelation and similar effects were observed with glycine, citrate and borate buffers (46). Chelation seems unlikely to explain the results obtained with salbutamol because it is not a characteristic of acetates, which stabilised the drug, but is of citrates and phosphates which had no stabilising effect. Experiments using different concentrations of buffers would help to clarify the mechanism because an increase in buffer concentration would be unlikely to increase the effect if chelation of contaminating ions is the sole mechanism. However, if the buffer salt exerts a specific catalytic action, an increase in concentration should accentuate the effect. Additional

evidence can be gained by examining the action of a powerful chelating agent such as EDTA.

Metal ions have been implicated in the oxidation of adrenaline (11, 34-36) but it is not certain whether they are essential for the initiation of the oxidation. Chaix *et al.* (11) found that in the absence of added metal ions, the rate of oxygen uptake was low and proposed that this was due to trace contamination. However, cyanide, which forms strong complexes with copper, did not prevent the oxidation of adrenaline and, in fact, caused a slight increase in oxidation rate (37). Other workers (109) have shown that although EDTA reduces the catalysis of adrenaline breakdown by metals, the extent of its effect depends on both the catalyst and the buffer used. Metal ions, particularly copper, are known to catalyse salbutamol degradation (12) and so some kinetic experiments were conducted in the presence of 0.05% EDTA. This should chelate any metal ions present as trace contaminants and thereby prevent metal-catalysed degradation.

The results, which are presented in Table 26, resemble those of Sokoloski and Higuchi (37) in showing an increased rate of reaction in the presence of the chelating agent. The implication is that the accelerating effect of phosphate could also have been due to chelation. Green *et al.* (38) observed a similar effect by iron on the rate of adrenaline breakdown. They showed that whilst iron alone did not alter the breakdown, the iron-EDTA complex was a powerful catalyst. They drew a parallel with the biological,

iron-containing catalysts of ferricytochrome and the ferritin complexes. In such systems the metal ion is held firmly in position, facilitating electron transfer during the catalytic process. A similar situation could exist with salbutamol but further work, involving the addition of metal ions to the reaction mixture is needed to confirm this.

Although the results discussed so far give indications of the probable routes of salbutamol degradation, conclusive evidence can only be obtained from the isolation and identification of breakdown products. Ideally, these compounds should then be assayed during kinetic experiments so that the relative importance of the various pathways and the existence of reactive intermediates can be established. Attempts were made, therefore, to isolate the major degradation products of salbutamol. However, the large number of compounds generated during the breakdown results in low concentrations of each individual product. It was therefore possible to isolate only one compound in sufficient quantity and purity for further study. This compound ran on TLC with a  $R_f$  value of about 0.75 and represented the largest spot, after salbutamol in the thin layer chromatogram.

This compound eluted from the HPLC column after the internal standard, AH 4045, suggesting that it was considerably less polar than salbutamol. Evidence from nuclear magnetic resonance spectroscopy (nmr) and mass

spectrometry (Section 3) whilst not conclusive, indicates a dimeric structure in which one amino side chain has been lost, as shown in Figure 55. It is possible that some of the other TLC spots represent higher polymers which eventually produce the yellow-brown precipitate which formed in extensively degraded salbutamol sulphate solutions.

Although polymeric products are formed as a result of adrenaline oxidation (29-31), the intermediates are indole derivatives, resulting from cyclisation of the catecholamine molecules. The reaction (Fig. 62) is believed to proceed via a semi-quinone radical which is rapidly oxidised further to an open-chain quinone (28, 30, 31, 37). This in turn probably undergoes oxidative intramolecular cyclisation to the corresponding aminochrome. It is possible that leuoadrenochrome (Fig. 62) is formed as an intermediate in the reaction (30, 31) but since it would rapidly lose water to form the 5,6-dihydroxyindole or be oxidised to adrenochrome, this mechanism is not certain. This reaction mechanism has been applied to many catechol derivatives including DOPA (110) and noradrenaline (30), and also to phenylephrine (39).

The aminochromes are highly coloured compounds and the formation of adrenochrome is the cause of the pink colour acquired by adrenaline solutions which are exposed to the air. However, they do not represent the end of the degradative process but can participate in polymerisation reactions to produce melanins. Bu'Lock (111) postulated

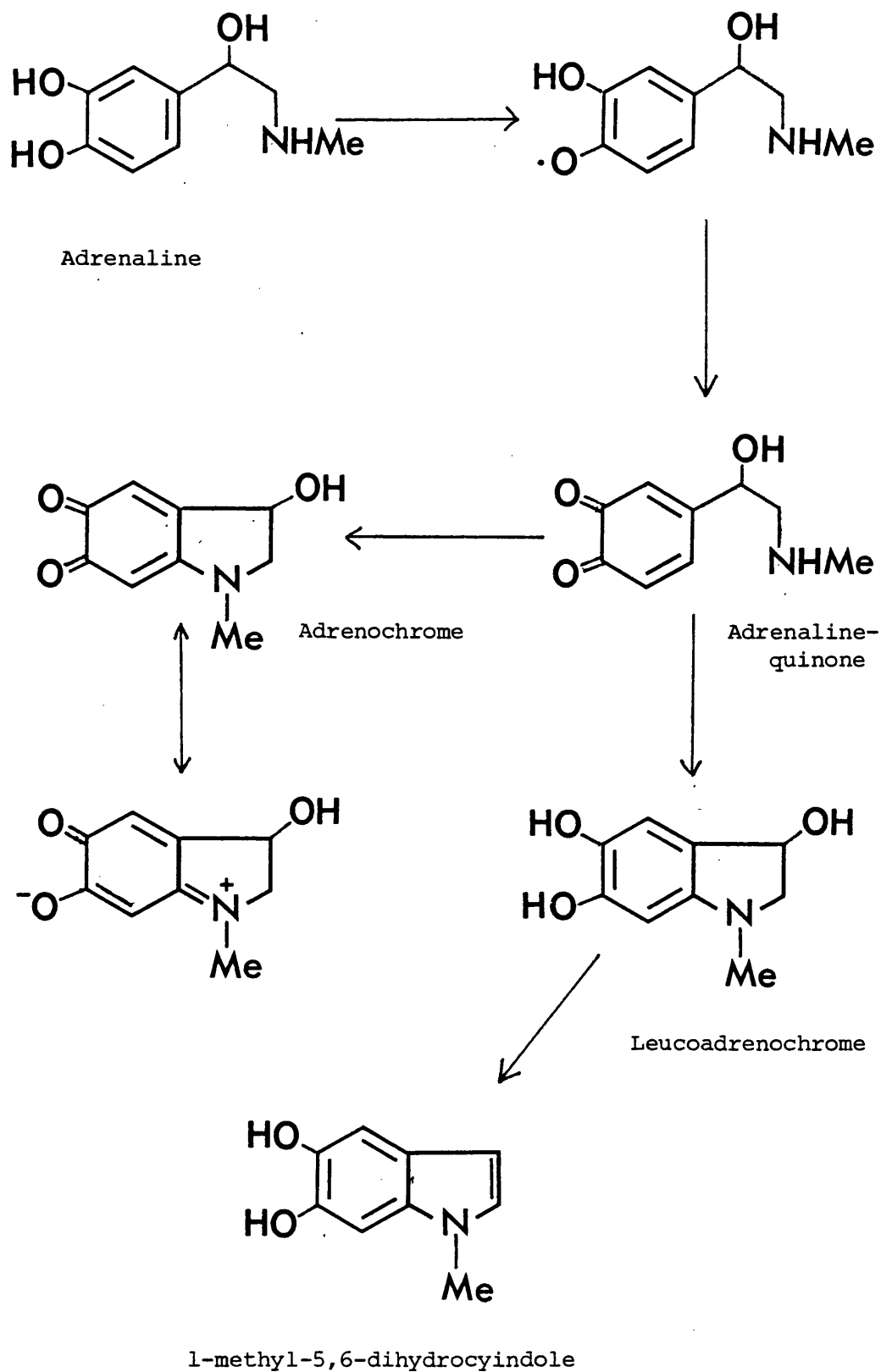


Fig. 62. The oxidation of adrenaline.

that the first step is a dimerisation which is acid catalysed. The dimer can then react further to produce the polymeric melanin (Fig. 63).

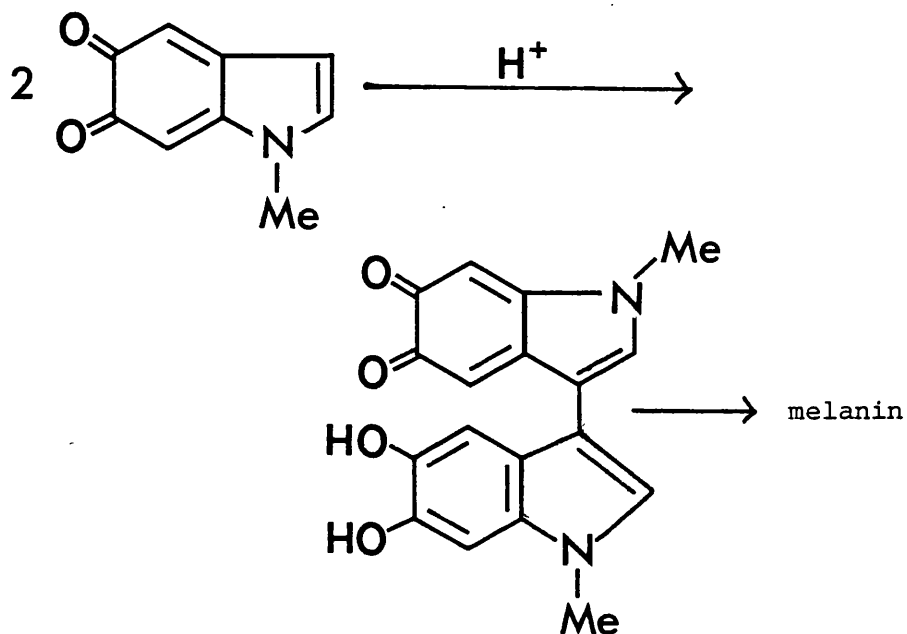


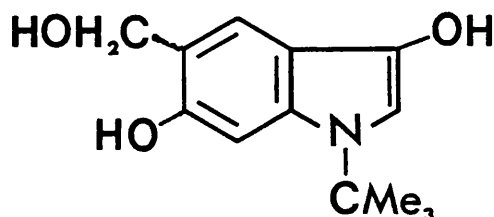
Fig. 63. The formation of melanin from adrenochrome

Bu'Lock believed the propagation reaction to occur between the dihydroxyindolyl group and the indoquinone of the monomer or other oligomers. The precise composition and structure of the polymer is variable, being dependent on the reaction conditions. Although the polymerisation can proceed in the absence of oxygen as shown by Harley-Mason (112), the product has a greater pyridine solubility, presumably due to a lower molecular weight or greater number of phenolic hydroxyls (111).

Although degraded salbutamol solutions formed a precipitate, this varied in colour from yellow to brown, unlike melanins which are typically dark brown or black (32). The proposed structure of the major decomposition



product (Fig. 55) suggests that, unlike catecholamines, salbutamol does not undergo intramolecular cyclisation to any significant extent. This is probably because salbutamol, by lacking the catechol group, cannot form an ortho-quinone which is a vital intermediate in the formation of aminochromes and the subsequent melanins (Fig. 63). The single phenolic group in salbutamol also results in a less highly activated ring, which would therefore be less sensitive to substitution reactions. However, when TLC plates of solutions which had been extensively degraded at high pH were examined under ultra-violet light, a small fluorescent blue spot was sometimes observed. It has been shown that some 5,6-dihydroxyindoles have a weak blue fluorescence (30) and so it is possible that the indole derivative of salbutamol is formed in small quantities under these extreme conditions but the reaction probably accounts for a very minor proportion of the



Possible structure of the indole derivative of salbutamol

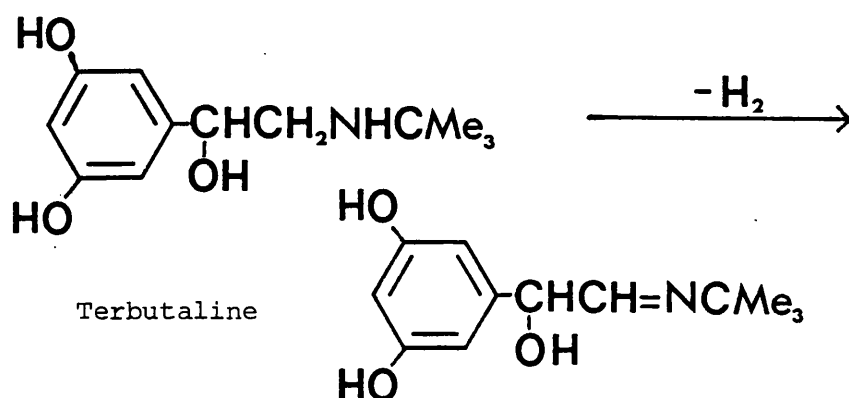
salbutamol loss.

The steric hindrance resulting from the bulky tertiary butyl group in salbutamol is also likely to cause differences between its reactions and those of the catecholamines. All

the natural catecholamines have small nitrogen substituents, being either primary amines (e.g. noradrenaline) or methyl substituted secondary amines (e.g. adrenaline), and so little hindrance is likely during the cyclisation reaction.

An alternative degradation pathway has been proposed for the sympathomimetic phenylephrine (75). The stability of this drug was studied at pH 6.8 and the workers concluded that the principal breakdown products resulted from a Pictet-Spengler reaction between the drug and formaldehyde generated during the degradation. The scheme they proposed is outlined in Fig. 64 and shows initial oxidation of the drug leading to the production of formaldehyde. This then reacts with further phenylephrine molecules to ultimately form isoquinoline derivatives.

Svensson (74) examined the stability of terbutaline, a bronchodilator similar in structure to salbutamol, and concluded that the Pictet-Spengler reaction constituted a minor degradation pathway. The major degradation product was identified as 1-(3,5-dihydroxyphenyl)-2-tertbutylimino ethanol, the result of a relatively simple oxidation.



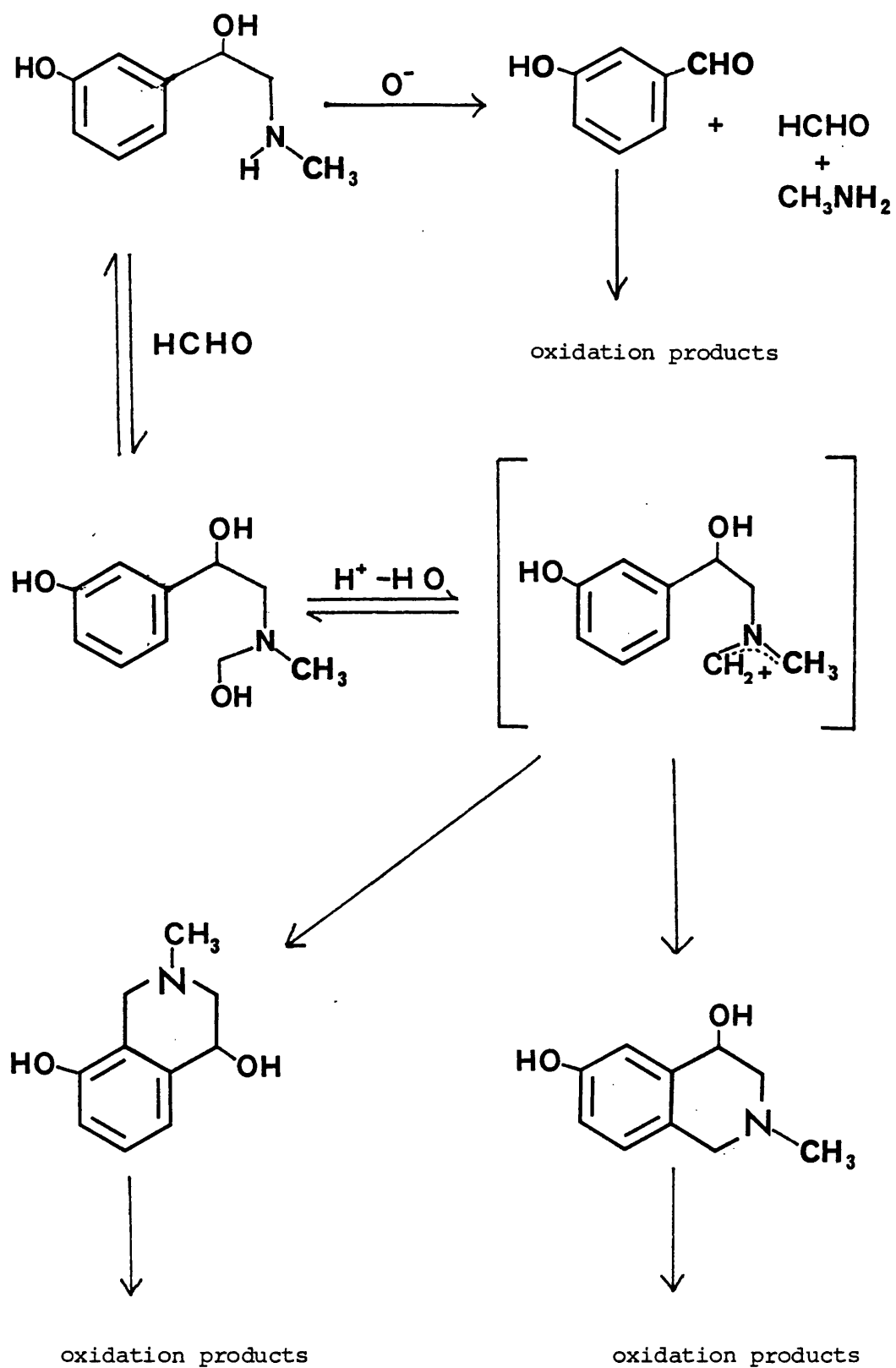


Fig. 64. The degradation of phenylephrine by the Pictet-Spengler reaction.

Bansal and Monkhouse, in their study of pirbuterol (42), also investigated the possibility of a Pictet-Spengler reaction. They added excess formaldehyde to a pirbuterol solution at pH 6 and examined the resultant solution by TLC. A new spot was observed in a position which did not correspond to any of the usual decomposition products and so they concluded that the pathway was not important in the normal breakdown of the drug.

Both phenylephrine and terbutaline have hydroxyl groups meta to the amino side chain. The mechanism of the Pictet-Spengler reaction, as depicted in Fig. 64 involves intramolecular electrophilic attack by the substituted amine. Since phenolic groups activate the ortho and para positions to such attack, both these drugs are candidates for the reaction. The lower reactivity of terbutaline, in spite of the dual activation by the two phenolic groups, is probably due to steric hindrance by the tertiary butyl substituent on the nitrogen atom. Salbutamol and pirbuterol are not able to cyclise in this way at an activated position on the ring because the phenolic groups are para-orientated to the side chain. These compounds are therefore unlikely to undergo the reaction and further discouragement to intramolecular reaction is provided by the tertiary butyl nitrogen substituent.

#### 4.4 The effect of sugars on the stability of salbutamol sulphate solutions

Although much more work is needed to establish the details of the salbutamol decomposition reactions, the influence of formulation excipients on the rate of breakdown can still be examined. Such studies are important during the formulation and development of a drug when the chemistry of the compound may still be under investigation.

Sugars were the principal excipients to be examined, since these are major ingredients in pharmaceutical syrups and have been shown to influence the degradation of some drugs (13, 16, 64-69). Fig. 44 shows that at pH 7.0, 10% glucose increased the degradation rate of salbutamol sulphate by a factor of 2 but sucrose had no effect at all. Fructose was not examined at this pH because the Lobry de Bruyn-van Ekenstein rearrangement (Section 1.6) results in the interconversion of glucose, fructose and mannose and so a solution of any one of these will, in fact be an equilibrium mixture of the three.

At pH 3.5, the region of maximum salbutamol stability in simple solutions, 50% glucose, fructose and sucrose increased the breakdown to similar extents (Fig. 45) resulting in initial apparent first order rate constants in the range of  $4 \text{ to } 5 \times 10^{-7} \text{ s}^{-1}$ . Reduction of the sucrose concentration to 10% caused a fall in the rate constant to  $5.47 \times 10^{-8} \text{ s}^{-1}$  and in the absence of sugars, no detectable breakdown of salbutamol occurred over a

similar time period (800 hours), as shown in Figure 4 6. The eight-fold increase in rate constant for a five-fold increase in sugar concentration indicates that the reaction order is greater than unity with respect to sucrose, but since the residual sugar concentration was not measured during these experiments, precise determination of the order was not possible.

It was surprising to find that the sugar concentration had such a pronounced effect on the rate of salbutamol degradation. Even at a concentration of 10% the monosaccharide molarity (0. 56 M) greatly exceeded that of the drug (0. 015M) and so would be expected to swamp the reaction which would then proceed at the maximum rate. The acceleration of salbutamol breakdown is unlikely to be due to changes in the dielectric constant, caused by the sugars, because sucrose would then be expected to be as effective as glucose and fructose at all pH values. Sugars reduce the dielectric constant of water and thus inhibit ionisation of solutes and ionic interactions. This parameter has been shown to affect reactions such as ester hydrolysis (135) but, like ionic strength, is less likely to affect reactions involving uncharged species. Changes in the dielectric constant would therefore not be expected to be the primary reason for the loss of stability of salbutamol sulphate in sugar solutions.

The results from the experiments using 50% sugar and citrate buffer at pH 3.5 showed that the buffer has little

effect on the rate of drug breakdown during the early stages although some stabilisation may occur once the residual drug concentration has fallen below 70% (Fig. 48). This is in keeping with the results obtained at pH 7.0 using citrate alone (Fig. 43), where it was noted that a reduction in the rate of reaction took place between 70% and 60% residual drug. The rate increased again below 60%, an effect which was not observed when 50% sugar was used with the citrate but this may have been masked by the sugar effects since even in the absence of citrate the rate of reaction decreased markedly as the drug concentration fell (Fig. 48). This slight stabilisation by citrate is of little practical significance in pharmaceutical formulation because it only manifests itself when considerable breakdown has already taken place. The aim in formulation is to devise a product which will degrade by no more than 10% during its shelf-life and so this citrate effect is of no apparent use.

The most significant feature of these experiments using sugars is the pH-dependence of the sucrose effect and this can be explained by considering the structure of the sugar. Sucrose is a disaccharide formed by a glycosidic linkage between a glucose unit and a fructose unit, as described in Section 1.6. This link involves the carbonyl group of both monosaccharides and is quite stable in neutral or alkaline solutions (57, 114). However, under acidic conditions hydrolysis occurs, liberating the constituent monosaccharides, glucose and fructose (113).

Equation (17) has been developed by Vukov (114) to describe the rate of sucrose hydrolysis under varying conditions of temperature, pH and concentration.

$$\log k = 16.91 + \log (d-c) - \frac{5670}{T} - \text{pH} \quad (17)$$

where  $k$  = hydrolysis rate constant ( $\text{min}^{-1}$ )

$d$  = density of solution ( $\text{g ml}^{-1}$ )

$c$  = sucrose concentration ( $\text{g ml}^{-1}$ )

$T$  = temperature

This equation was used to calculate the extent of sucrose hydrolysis under the experimental conditions, and the results are summarised in Table 40.

| Conditions         | $k$ ( $\text{min}^{-1}$ ) | Half-life |
|--------------------|---------------------------|-----------|
| 50% sucrose pH 3.5 | $5.08 \times 10^{-4}$     | 23 hours  |
| 10% sucrose pH 7   | $4.56 \times 10^{-7}$     | 2.9 years |

Table 40. The rate of sucrose hydrolysis under varying conditions of pH and concentration at  $70^{\circ}$

The effect of this difference in hydrolysis rates is profound since at pH 7.0, less than 0.1% hydrolysis will occur during the course of the experiment (250 hours) at  $70^{\circ}$ . However, the more rapid reaction at pH 3.5 means that the sugar will be extensively hydrolysed at an early stage of salbutamol breakdown as illustrated in Figure 65. The interaction between salbutamol and sucrose



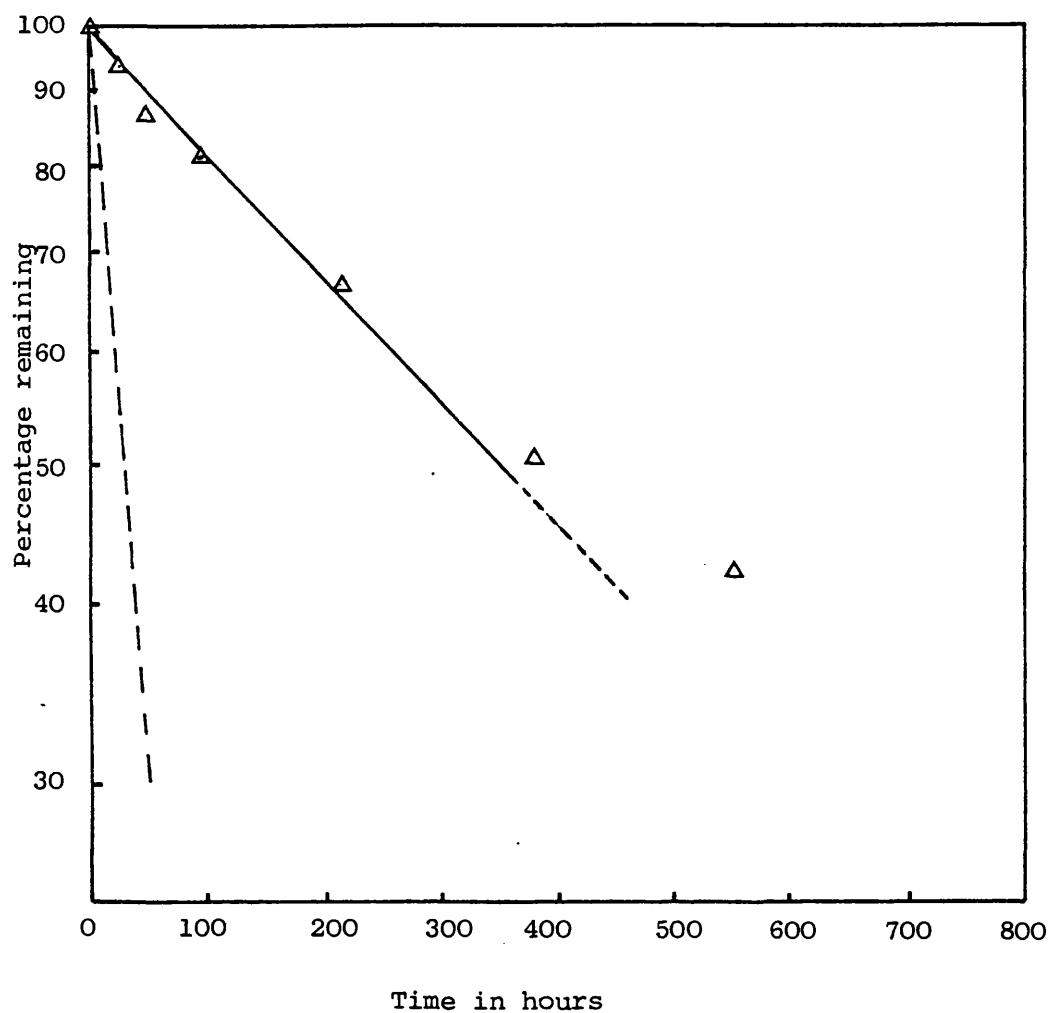


Fig. 65. Comparison of hydrolysis rate of sucrose (dotted line) with degradation rate of salbutamol sulphate ( $\Delta$ ) in solution containing 50% sucrose and 0.5% salbutamol sulphate at  $70^{\circ}$  and pH 3.5.

is therefore dependent on the hydrolysis of the sugar to its constituent monosaccharides. These differ from the disaccharide in having a potentially "free" carbonyl group in their open chain or acyclic form. The drug-sugar interaction may therefore take place at this carbonyl which in sucrose is absent, being used in the formation of the glycosidic bond.

The differences between the effects of the sugars, which became apparent after approximately 300 hours at 70° and pH 3.5 (Fig. 45) are probably due to differences in the stability of the sugars themselves. The reaction rate in the presence of fructose slowed down more quickly than in the presence of the other two sugars and may reflect the greater tendency of fructose to form furan derivatives (58, 113).

The samples from all the sugar experiments, except that of sucrose at pH 7.0, showed an additional peak in the HPLC trace with a  $k'$  value of approximately 0.4 (Fig. 47). This implied that the increase in the degradation rate was due to an additional specific reaction between the drug and the sugar resulting in a new breakdown product. TLC examination of the degraded solutions revealed an additional spot at high  $R_f$  in the samples from experiments at pH 3.5 (Fig. 66). At pH 7.0 the picture was not so simple because the drug had degraded extensively by the normal routes and the resultant spectrum of breakdown products obscured the presence of additional spots (Fig.

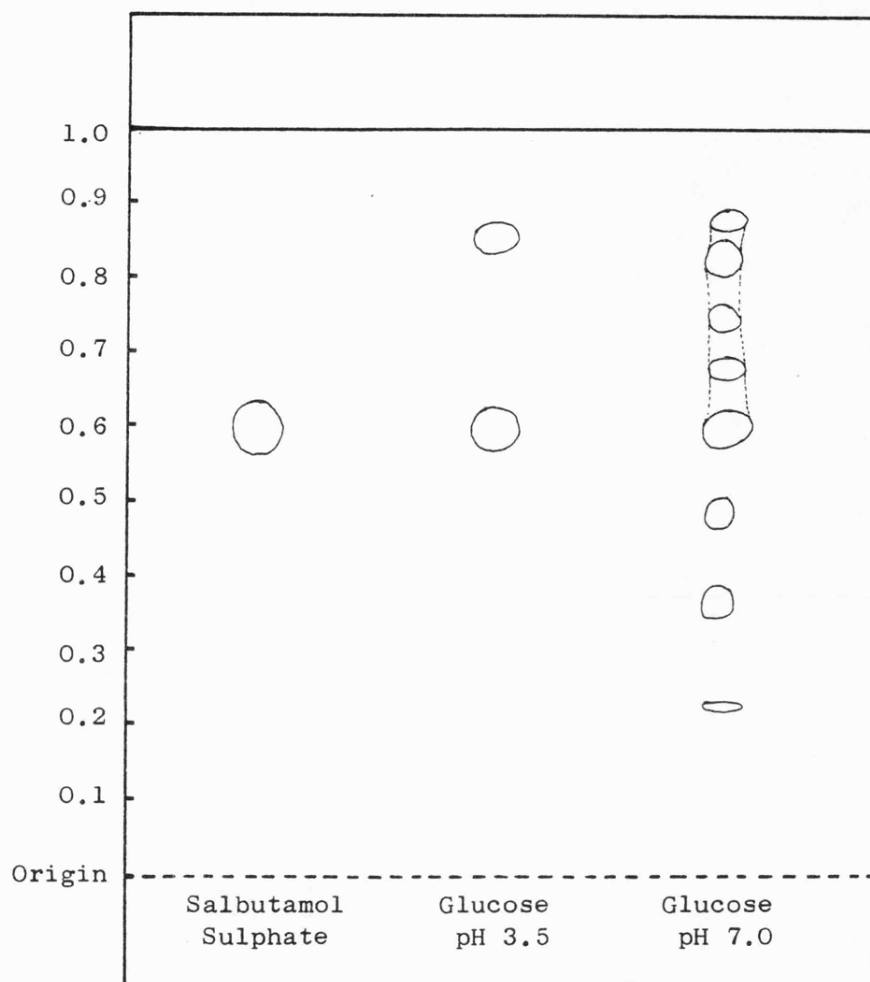
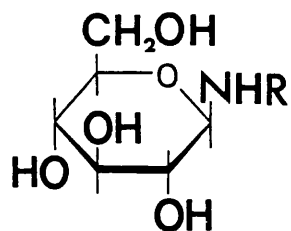
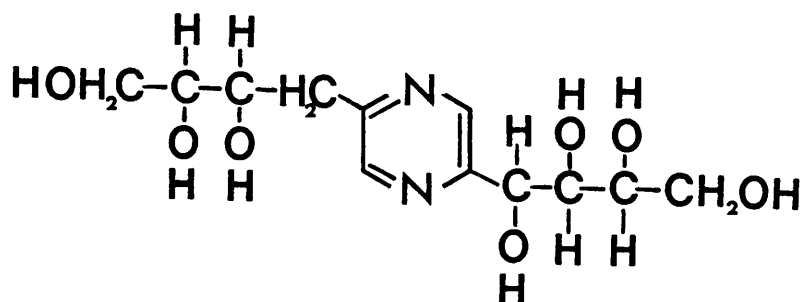


Fig. 66. Diagrammatic TLC plate of a standard 0.5% salbutamol sulphate solution, together with samples degraded in the presence of glucose at pH 3.5 and pH 7.0 at 70°.

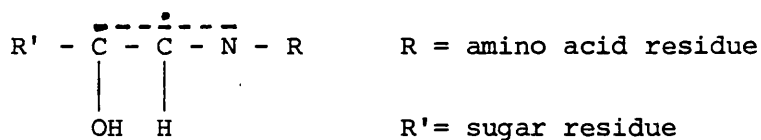
66). This did not happen at the lower pH because the degradation of salbutamol sulphate in simple solution at this pH is so slow that spots due to these reactions were very small, the major degradation being due to the sugar interaction.

Interactions between glucose and ammonia or amines have been reported on several occasions (121-125), the products usually being glucosylamines. Mitts and Hixon (121) prepared several glucosyl-n-alkylamines by heating the sugar and amine at 70 ° with 0.5N hydrochloric acid. These conditions are similar to those used in the kinetic experiments with salbutamol and mildly acidic conditions have also been used by Japanese workers to prepare the N-glucoside of sulphanilamide (127). They used a pH of 3-4 and found that the yield of glucoside was much greater when the concentration of sugar exceeded that of the amine. Although most workers have used simple primary amines, secondary amines are reported to give similar products (126), the reaction often being slower in these cases. Glycosylamines hydrolyse quite readily (121-123), the ease of reaction being related to the basicity of the amine (121). Both dilute acids and alkalis can catalyse the hydrolysis, their relative effectiveness being dependent on the compound in question (123). A different compound, deoxyfructosazine has been isolated as a product of the reaction between glucose and ammonia at pH 5.3-6.0 (124).

 $\beta$ -D-glucosylamine

Deoxyfructosazine

The mechanism of the reaction between sugars and amino acids has been studied by Namiki and Hayashi (125) using electron spin resonance spectroscopy (ESR). Although sugar solutions alone did not produce free radicals, addition of amino acids to the solutions caused radical formation. The conclusion was that radicals are developed early in the amino-carbonyl reaction and the suggested structure was that of an enaminol, resulting from the rearrangement of the glycosylamine.



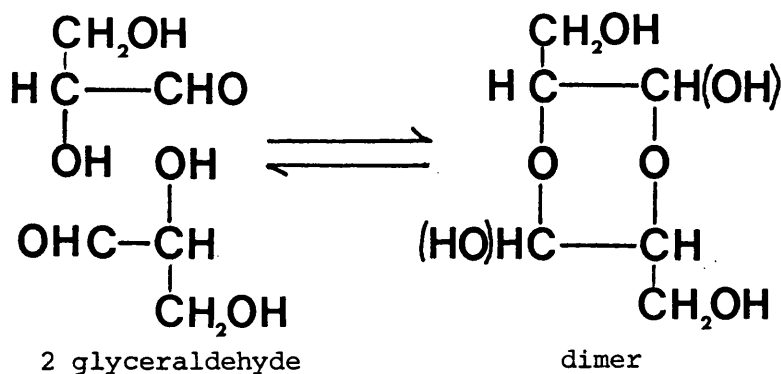
In order to test this hypothesis that the carbonyl is the site of interaction between sugars and salbutamol, glyceraldehyde, a simple, non-volatile aldehyde was studied.

The result, depicted in Figure 51, indicated a lag phase followed, after approximately 50 hours, by a rapid acceleration of the degradation. This implies that the aldehyde is not active as such but requires a preliminary reaction before it can exert an effect on salbutamol. In their studies on sugars and amino acids, Namiki and Hayashi (125), were able to detect radicals immediately but chemical reaction, indicated by browning of the reaction mixture, often took longer to become apparent. It is possible that in the reaction between salbutamol and aldehydes, time is needed for the free radicals to accumulate to a concentration which will initiate reaction. Such a lag phase has been observed in the oxidation of catecholamines and ascribed to the formation of radicals (28). Once the highly reactive radicals have reached the "critical level", a chain reaction is initiated and loss of drug becomes apparent. This lag phase was not observed in the experiments with sugars but this could be due to their greater concentration (50% instead of 5%) which would enable radicals to be generated more quickly. However, this higher concentration of excipient would also increase the possibility of a radical reacting with a sugar molecule instead of the drug and this could explain the slower loss of salbutamol from such solutions, compared to that from the glyceraldehyde solution.

An alternative possibility is that the glyceraldehyde is oxidised to glyceric acid which then reacts with salbutamol. Consequently, a similar experiment using

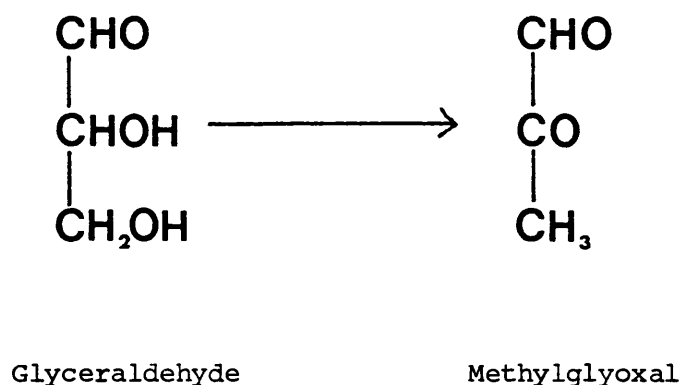
glyceric acid instead of glyceraldehyde was performed. Although the results from this experiment revealed a rapid loss of salbutamol during the early stages of the reaction (Fig. 51), the rate fell dramatically after about 50 hours when about 70% salbutamol sulphate remained. It is most unlikely that this represents depletion of the glyceric acid because the 5% (0.47 M) concentration used was greatly in excess of that of the drug (0.015 M). Oxidation of glyceraldehyde to glyceric acid is therefore probably not the mechanism of its reaction with salbutamol.

Glyceraldehyde can form a dimer by intermolecular acetal formation (61) but the resultant compound has a cyclic structure analogous to that of the hexoses.



It has already been shown that the hexoses do not react with salbutamol when the carbonyl group is unavailable, as in sucrose, and so the glyceraldehyde dimer is not a credible intermediate in the salbutamol reaction.

However, another compound obtainable from glyceraldehyde is methylglyoxal, the production of which from glyceraldehyde has been shown to be catalysed by amines (128, 129).



Methylglyoxal is a highly reactive compound and polymerises readily (129). If formed in the reaction mixture containing salbutamol it would therefore be a likely intermediate in salbutamol breakdown and would account for the rapid loss of drug after an initial induction period.

None of the proposed mechanisms for the aldehyde-drug reaction explain the effect of glyceric acid, however..The evidence points to depletion of the active compound but since the acid itself is present in such excess, a contaminant seems to be indicated. Approximately 30% of the salbutamol sulphate in the solution was used in the reaction, which represents a concentration of 0.15%.

$$0.15\% \text{ salbutamol sulphate} \equiv 0.446 \times 10^{-2} \text{ M}$$

$$5\% \text{ glyceric acid} \equiv 0.472 \text{ M}$$

Assuming that the contaminant reacts 1:1 with salbutamol sulphate, on a molar basis, the contamination level would need to be:

$$\frac{0.446 \times 10^{-2}}{0.472} \times 100 = 0.94\%$$



Since the glyceric acid was not of Analar grade, a purity level of 99% seems quite reasonable and so the reaction with salbutamol need not have involved the excipient under examination. The nature of the possible impurity is not clear from these results. No lag phase was detected and so glyceraldehyde is unlikely to be the responsible component but further work is needed to identify alternatives.

Since neither the aldehyde grouping, nor its oxidation derivative, the carboxylic acid appeared to be directly responsible for the degradation of salbutamol, alternative mechanisms were examined. Sugars are themselves subject to degradation, particularly at alkaline pH (115) 5-hydroxymethylfurfural (5-HMF) being a characteristic product (58, 59, 106-108), together with various organic acids (54, 116, 117) and lower molecular weight sugars (118). The effect of concentration on the rate of glucose decomposition appears to vary with the temperature used. Zheltukhin (117) found the rate constant in aqueous solution to be independent of concentration at 167° but to increase with the concentration at 186°. He ascribed this increase to autocatalysis by formic acid, a breakdown product at these extreme temperatures. Webb *et al.* (107) using temperatures around 100°, showed glucose solutions to be more stable at higher concentrations. If this is the case, and the loss of salbutamol is due to sugar decomposition products, the rate of drug loss should not be markedly affected by the sugar concentration. It was shown though, that the disappearance of salbutamol was much slower in the presence

of 10% sucrose than in the presence of 50%.

Having demonstrated from the experiment with glyceric acid (Section 2.12k) that carboxylic acids are unlikely intermediates in the reaction, the only decomposition product likely to have a significant effect was 5-HMF. This is a reactive compound which is believed to polymerise readily and to be responsible for much of the discolouration of degraded sugar solutions (58, 59, 120). It is produced by the dehydration of the sugar molecules, usually under acidic conditions and its appearance has been used to follow the decomposition of glucose solutions (59, 107, 108). Nevertheless, the amount of 5-HMF produced by glucose is quite small (59) although greater yields are obtained from fructose solutions (58, 113). If the sugar-initiated loss of salbutamol is mediated by 5-HMF, greater loss would therefore be expected from fructose solutions than from glucose or sucrose solutions. Figure 45 shows however, that there were no significant differences in the initial rates, and that beyond approximately 35% degradation, salbutamol was more stable in the presence of fructose than in glucose or sucrose solutions. These results therefore do not lend support to this theory. However, in order to resolve the question, 5-HMF was tested at concentrations likely to result from the degradation of sugar solutions and was found to have no effect on the rate of salbutamol breakdown, as illustrated in Table 30. This provides further evidence that a carbonyl group is not the sole requirement for the sugar-drug interaction.

It appeared, therefore, that the acceleration of salbutamol breakdown by sugars is not due to sugar decomposition products, nor to a direct reaction between the sugar carbonyl and the drug. It seemed unlikely that a reaction occurs between the sugar hydroxyls and the drug because sucrose did not accelerate salbutamol degradation until hydrolysed although hydroxyl groups are available for reaction in the disaccharide. The hydroxyl group in 5-HMF is also very reactive (120) but this compound had no detectable effect on the rate of salbutamol breakdown. However, in order to establish the role, if any, of this functional group in salbutamol degradation, the polyhydric compounds propylene glycol, glycerol and sorbitol were studied under various conditions.

All three excipients increased the rate of salbutamol degradation but to varying extents. Propylene glycol and glycerol were used at a concentration of 10% at pH 7.0 and the initial rate of breakdown in both cases was faster than that in a simple salbutamol sulphate solution at pH 7.0 (Fig. 49). In the case of the dihydric alcohol, the rate constant was increased by more than 100% at first but this fell after approximately 50 hours and then approximated to that in a simple solution. Like the glyceric acid result, this cannot be due to depletion of the main component because it is present in great excess over the drug. However, small amounts of impurities may be responsible for the initial rapid loss of drug, the rate of which was slightly greater than that resulting from the addition of

10% glucose (Table 27).

The rate in the presence of the trihydric alcohol, glycerol, did not display this tendency to fall dramatically after a relatively short time. The increase in the rate constant was less than that obtained in the presence of glucose, being approximately 33% over that for a simple solution and was constant throughout the experiment, suggesting a reaction between salbutamol and glycerol rather than an impurity in the excipient.

The HPLC traces revealed an additional peak in both solutions but TLC examination was inconclusive because of the masking effect of the normal degradation products, as occurred with sugar solutions at pH 7.0. The product formed in the presence of propylene glycol eluted from the HPLC column between salbutamol and the internal standard but the glycerol reaction product eluted so close to salbutamol that it was only resolved when applied to a column of higher plate number. This order of elution reflects the greater polarity of glycerol, with three hydroxyls, over propylene glycol with two. This trend was continued in the studies using 50% polyhydric alcohol (sorbitol) at pH 3.5. The sorbitol reaction product eluted even earlier, before salbutamol, in a similar position to that from the hexose solutions but having a slightly higher capacity factor (1.0 instead of 0.4). The additional peak in the glycerol solution at pH 3.5 was in the same place as that at pH 7.0. being barely separated from salbutamol. The presence of

a new compound in these solutions was confirmed by TLC examination of the solution at pH 3.5 when an extra spot, well separated from salbutamol, was clearly visible.

The kinetic results at pH 3.5 (Tables 28 and 29) revealed that the polyhydric alcohols and the sugars are similar in their effects on salbutamol degradation although the initial rate constant from the sorbitol experiment was statistically different from those obtained in the presence of sugars. Since the graphs are all curved (Figs. 45 and 50), the significance of initial rates is equivocal because they are dependent on the number of data points selected in their calculation. The similarity of the effects of sugars and polyhydric alcohols under these conditions is surprising in the light of the previous evidence concerning sugars; the lack of sucrose effect at pH 7 (Fig. 44) suggesting that hydroxyl groups, and therefore polyhydric alcohols would have little effect on salbutamol degradation. Although the reaction mechanisms may differ between the two classes of compound, the similarity of the rate constants and the appearance of a peak in the HPLC traces of the sorbitol solution, comparable to that from sugar solutions, implies that the reactions are closely related, if not identical.

Kitson *et al.* (16, 17) have postulated the oxidation of polyhydric alcohols to reducing sugars in order to explain their effect on the stability of a triazolo-pyrimidine derivative. They found that whilst methanol and

acetone had no effect on the degradation rate of the drug, acetaldehyde and reducing sugars markedly increased the rate of decomposition. The polyhydric alcohols, mannitol and sorbitol, were effective only in the presence of oxygen and solutions of these alcohols which had been heated at 100° for a few days gave positive results with Fehling's reagent, indicating the presence of reducing compounds. The decreased stability of the drug in the presence of reducing sugars was ascribed to the formation of Schiff bases (Section 1.7).

During the studies on salbutamol stability it was noted that solutions of salbutamol sulphate, both alone and in the presence of sugars, discoloured markedly as degradation proceeded, usually resulting in the formation of a yellow-brown precipitate. Sugar solutions discoloured particularly quickly, presumably due to decomposition of the sugar itself. However, in the presence of polyhydric alcohols the solutions remained colourless, even when the residual salbutamol sulphate concentration had fallen to less than 50%. This implies that extensive oxidation of the polyol to reducing sugars, as suggested by Kitson et al. (16, 17) does not take place, since the sugars so formed should degrade in the normal way.

The only way to be certain of the nature of the degradation products formed in the presence of syrup excipients is to isolate and identify them. Attempts by the methods described in Section 2.7, to isolate the adduct produced by the reaction

with glucose resulted in a compound whose nmr spectrum was virtually the same as that of salbutamol. When subjected to thin layer chromatography the compound ran as salbutamol, having an Rf of about 0.6 instead of the previous 0.85. The conclusion drawn from this was that hydrolysis of the adduct had taken place during the extraction and purification process. If the compound was a glucosylamine, evidence from published data (121-123) indicates that hydrolysis would take place readily and so careful isolation procedures would be necessary to maintain its integrity. However, the ease of hydrolysis may also mean that the reaction between the drug and glucose is of little pharmacological significance. If this took place in a pharmaceutical product, such as an oral syrup, and yet the salbutamol was liberated in the gastro-intestinal tract, the bioavailability of the formulation may not be affected.

The reaction in the presence of 50% glycerol at pH 3.5 resulted in a compound which was more stable and could be isolated successfully. The analysis of the nmr spectra of the compound is described in Section 3, and a possible structure is depicted in Figure 57.

#### 4.5 Conclusions

This work has served as an introduction to the stability of salbutamol sulphate solutions, indicating the major factors influencing the stability of the drug but, as yet,

unable to fully explain the effects or propose mechanisms for the drug's reactions. The work was hindered to some extent by the slow rate of degradation under some conditions which precluded the replication of many experiments. A particular example is the experiment at pH 5.0 using a drug concentration of 0.5% at 70<sup>o</sup>, performed during the investigation of the pH-stability profile (Table 24). After running for 450 hours this experiment had to be stopped because of a water bath malfunction and an opportunity to repeat it did not arise. The rate constant obtained is therefore based on only 10% degradation of the drug and is thus subject to a greater degree of uncertainty than those which were obtained from more extensively degraded solutions. The use of higher temperatures would reduce the time needed for each experiment but introduce the possibility of initiating atypical reactions which do not represent those prevailing at more normal temperatures.

Non-isothermal methods allow the temperature to be increased over the course of the experiment, reducing the time needed and these may prove useful in the study of salbutamol sulphate. However, the Arrhenius plot at pH 7.0 would need to be checked first because a linear Arrhenius relationship is necessary if non-isothermal methods are to produce valid results.

Further work which is suggested by the results obtained so far include an Arrhenius study at about pH 3, near the region of maximum salbutamol sulphate stability and therefore



the region of formulation. The influence of metal ions and chelating agents appears to require further investigation in order to explain the unusual behaviour of EDTA in accelerating the decomposition of the drug. Similarly, the effects of buffer salts could be examined at different concentrations to establish whether their action is exerted via chelation or direct catalysis.

One of the most interesting aspects of this work was the effect of sugars on the breakdown of the drug. The absence of an accelerating action by sucrose at pH 7.0 suggested at first that this might be a better pH for the syrup formulation than the current one of 3.5. However, even in the presence of 50% sugar at pH 3.5, the rate of salbutamol degradation is less than that in a simple solution at pH 7.0. Further investigation of the concentration dependence of the sugar effects would be useful since it is likely that a limiting value is reached somewhere between 10% and 50%, the only two concentrations examined so far. An Arrhenius relationship in the presence of sugar would also be of value and may help in establishing the nature of the drug-sugar reaction.

Additional work on sugar chemistry, particularly that relating to the oxidation of polyhydric alcohols would help to explain the action of these compounds on the rate of breakdown of the drug. The disaccharide, maltose, would provide further evidence relating to the requirement for a "free" carbonyl in the salbutamol-sugar interaction.

This sugar, unlike sucrose, has only one carbonyl bound into the glycosidic link and if studied at pH 7.0 would be expected, from the evidence so far, to accelerate the degradation of the drug. At pH 3.5 maltose, like sucrose is hydrolysed to its constituent monosaccharides.

Finally, development of expertise in isolating and identifying the major breakdown products is needed. The results obtained in this study were disappointingly inconclusive and further purification of the isolated materials is needed in order to identify them with any degree of certainty. The reactivity of the compounds require the mildest possible conditions to be used at all stages of their isolation if further decomposition is to be prevented.

#### 4.6 Pharmaceutical Implications

Since this work was concerned with the stability of aqueous solutions of salbutamol sulphate and the effect of formulation excipients on this, it would be useful to make some tentative predictions of the shelf life of salbutamol products. All the work was conducted at temperatures far in excess of normal storage conditions and in many cases the pH was not pharmaceutically realistic for reasons of experimental convenience. However, some cautious extrapolation of the results is possible.

From the pH-stability profile of the drug at 70° (Fig.

40), the rate constant at pH 3.5 is predicted to be approximately  $6.0 \times 10^{-9} \text{ s}^{-1}$ . Assuming that the same Arrhenius relationship holds at this pH as at pH 7 and 9, then, from equation (8)

$$\log k = \log A - \frac{E_a}{2.303RT}$$

$$\therefore \log k_{25} - \log k_{70} = \frac{-E_a}{2.303R} \left( \frac{1}{298} - \frac{1}{343} \right)$$

$$\therefore \log k_{25} - \log (6.0 \times 10^{-9}) = \frac{-107 \times 10^3}{2.303 \times 8.314} (4.4 \times 10^{-4})$$

$$\begin{aligned} \log k_{25} &= -2.46 - 8.222 \\ &= -10.682 \end{aligned}$$

$$\therefore k_{25} = 2.08 \times 10^{-11} \text{ s}^{-1}$$

$$\begin{aligned} t_{10\%} &= \frac{0.105}{k} \\ &= 5.07 \times 10^9 \text{ s} \\ &= 163 \text{ yrs} \end{aligned}$$

It would seem therefore that a 0.5% solution of salbutamol sulphate at pH 3.5 would be perfectly stable under normal storage conditions.

The safety margin at this pH appears to be more than adequate and so formulation at a higher pH, nearer to physiological values should be possible. If a shelf-life of three years is selected then,

$$k_{25} = \frac{0.105}{36 \times 30 \times 24 \times 3600}$$

$$k_{25} = 1.13 \times 10^{-9} \text{ s}^{-1}$$

$$\log k_{25} - \log k_{70} = \frac{-E_a}{2.303R} \left( \frac{1}{298} - \frac{1}{343} \right)$$

$$\log k_{70} = \log (1.13 \times 10^{-9}) + 2.46$$

$$\log k_{70} = 2.46 - 8.95$$

$$k_{70} = 3.26 \times 10^{-7} \text{ s}^{-1}$$

From Fig. 40, such a rate constant would result from formulation at about pH 6. Since oxygen was found to markedly increase the degradation rate of the drug (Table 19, Fig. 35), the shelf life could be extended by packing the solution in ampoules under a gas such as nitrogen.

If a similar calculation is performed, this time with the more tenuous assumption that the activation energy for the drug-sugar reaction is the same as that for the degradation in simple solution, an estimation of the shelf-life of the oral syrup may be made.

$$k_{70} = 4.81 \times 10^{-7} \text{ s}^{-1} \quad (\text{Table 28})$$

$$\log k_{25} - \log (4.81 \times 10^{-7}) = \frac{-107 \times 10^3}{2.303R} (4.4 \times 10^{-4})$$

$$\log k_{25} = -2.46 - 6.318$$

$$= -8.778$$

$$\therefore k_{25} = 1.67 \times 10^{-9} \text{ s}^{-1}$$

$$t_{10\%} = \frac{0.105}{k}$$

$$= 6.3 \times 10^7 \text{ s}$$

$$= 24.3 \text{ months}$$

A syrup formulation containing 0.5% salbutamol sulphate would therefore be predicted to have a shelf-life of barely two years.

Obviously these extrapolations are highly unreliable and much more work would be needed to produce more relevant data but they serve to illustrate at least, the profound effect formulation excipients can have on the shelf-life of a drug.

## APPENDIX 1 STATISTICAL METHODS

### Least squares regression analysis

If two variables  $x$  and  $y$ , bear a linear relationship to each other, the best way of determining the equation of the straight line is by least squares analysis. This fits the best line through a set of points by minimising the sum of the squares of the deviations of the points from the line. The general equation of the line is  $y = a + bx$  and the values of  $a$  and  $b$  may be determined from the following formulae:

$$b = \frac{\sum (x - \bar{x})(y - \bar{y})}{\sum (x - \bar{x})^2}$$

$$a = \bar{y} - b\bar{x}$$

where  $\bar{y}$  and  $\bar{x}$  are the mean values of  $y$  and  $x$  respectively.

The correlation coefficient,  $r$ , is a measure of how well the data fit a straight line relationship and may be determined from the formula:

$$r = \frac{\sum (x - \bar{x})(y - \bar{y})}{\sqrt{\sum (x - \bar{x})^2 \sum (y - \bar{y})^2}}$$

The variance of the slope,  $b$ , is termed  $\sigma_b^2$  and is given by the equation

$$\sigma_b^2 = \frac{\sigma^2}{\sum (x - \bar{x})^2}$$

where  $\sigma^2 = \frac{\sum \epsilon^2}{n-2}$  and  $\epsilon = y - (a + bx)$

The variance of  $a$ ,  $\sigma_a^2 = \sigma^2 \left[ \frac{1}{n} + \frac{\bar{x}^2}{\sum (x - \bar{x})^2} \right]$

The standard deviations of  $a$  and  $b$  are equivalent to the square root of the variance. Details of the derivation of these formulae may be found in standard statistical texts (140).

Student's t-test

This is used to compare two estimates of a parameter,  $p_1$  and  $p_2$ , having standard deviations  $\sigma_1$  and  $\sigma_2$  respectively. The value of  $t$  is calculated according to the formula:

$$t = \frac{p_1 - p_2}{\sqrt{\sigma_1^2 + \sigma_2^2}}$$

This value is then compared with tabulated values of  $t$  (141) having  $N-4$  degrees of freedom where  $N$  is the sum of the number of observations used in determining  $p_1$  and  $p_2$ .

If the calculated value of  $t$  exceeds the tabulated value at the 5% probability level, the two estimates are considered to be significantly different.

Bartlett's Test

This is similar to Student's  $t$ -test but is used when more than two estimates of a parameter are being compared.

$$B = \frac{\sum (p_1 - \bar{p})^2}{\hat{\sigma}_2}$$

where

$$\hat{\sigma}_2 = \frac{n_1 \sigma_1^2 + n_2 \sigma_2^2 + \dots + n_n \sigma_n^2}{n_1 + n_2 + \dots + n_n}$$

$p_1, p_2$  are the estimates of the parameter, having standard deviations  $\sigma_1, \sigma_2$  etc. respectively and  $n_1, n_2$  etc. are the numbers of observations used in making each estimate of  $p$ .

$B$  will have a  $\chi^2$  distribution if the estimates of  $p$  come from

a normal distribution and so the calculated value of B may be compared with tabulated values of  $\chi^2$  (141) having N-1 degrees of freedom. (N is the number of estimates of p). As with t, if the calculated value exceeds the tabulated value at the 5% probability level, the estimates of p may be considered to differ significantly.



Appendix 2. Slopes of HPLC calibration lines over the course of a year

| Date   | Slope | Date    | Slope | Date     | Slope |
|--------|-------|---------|-------|----------|-------|
| 4.1.79 | 10.12 | 27.6.79 | 9.11  | 19.10.79 | 8.94  |
| 8.1    | 10.21 | 4.7     | 9.72  | 23.10    | 8.83  |
| 29.1   | 10.26 | 17.7    | 9.91  | 25.10    | 9.01  |
| 19.2   | 10.79 | 25.7    | 10.01 | 29.10    | 9.09  |
| 21.2   | 10.28 | 26.7    | 10.33 | 30.10    | 9.08  |
| 22.2   | 10.54 | 31.7    | 10.00 | 1.11     | 9.00  |
| 27.2   | 11.38 | 1.8     | 10.46 | 6.11     | 8.89  |
| 5.3    | 9.26  | 2.8     | 10.25 | 8.11     | 9.07  |
| 12.3   | 9.45  | 14.8    | 10.38 | 13.11    | 9.20  |
| 13.3   | 9.27  | 15.8    | 10.29 | 16.11    | 8.88  |
| 14.3   | 9.52  | 31.8    | 9.06  | 20.11    | 9.20  |
| 21.3   | 9.15  | 3.9     | 9.18  | 22.11    | 8.87  |
| 26.3   | 9.51  | 4.9     | 9.20  | 26.11    | 9.38  |
| 28.3   | 9.40  | 20.9    | 9.22  | 27.11    | 8.77  |
| 5.4    | 9.86  | 25.9    | 9.64  | 29.11    | 9.28  |
| 2.5    | 10.18 | 27.9    | 8.97  | 30.11    | 9.38  |
| 8.5    | 9.73  | 2.10    | 9.01  | 7.12     | 9.03  |
| 22.5   | 9.57  | 5.10    | 9.10  | 14.12    | 9.18  |
| 30.5   | 9.24  | 9.10    | 9.23  | 19.12    | 9.18  |
| 4.6    | 9.13  | 12.10   | 9.10  |          |       |
| 26.6   | 8.88  | 16.10   | 9.18  |          |       |

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